Production of Standardized Biofilms with Isolates from Household Washing Machines

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Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Jasmin Claudia Gattlen

von

Bürchen VS

Promotionskomitee

Prof. Dr. Leo Eberl (Vorsitz)

Dr. Laurie Mauclaire (Leitung der Dissertation)

Prof. Dr. Jakob Pernthaler

Dr. Manfred Zinn

Prof. Dr. Jan Roelof van der Meer (Externer Gutachter)

Zürich, 2012

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List of abbreviations

AFM	Atomic force microscopy
AHL	Acyl-homoserine lactone
ANOVA	Analysis of variance
BSA	bovine serum albumin
CDC	Centers for Disease Control
CDFF	Constant depth film fermenter
CFU	Colony forming units
CLSM	Confocal laser scanning microscopy
CTC	5-cyano-2,3-tolyl-tetrazolium chloride
CV	Crystal violet
dc	Dye-casted
DMSO	Dimethylsulfoxide
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
EPS	Extracellular polymeric substances
ESEM	Environmental scanning electron microscopy
GSM	Gym streptomyces medium
IEC-A*	Standard washing detergent consisting of IEC-A base, Na-perborate and TAED
INT	(2- (4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride
KBE	Kolonie bildende Einheit
LPS	Lipopolysaccharide
MDR	Modified Robbins device
MIC	Minimal inhibitory concentration
NB	Nutrient broth
n. d.	not determined
OD	Optical density
p. i.	post inoculation
PIA	Polysaccharide intercellular adhesin
PE-1000	Polyethylene with very high molecular weight
PP	Polypropylene

QACs	Quaternary ammonium compounds
QS	Quorum sensing
RAB	Rotating annular reactor
RDR	Rotating disk reactor
rpm	Revolution per minute
SEM	Scanning electron microscopy
TAED	Tetra acetyl ethylene diamine
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSY	Trypticase soy yeast extract medium
SDA	Sabouraud dextrose agar
SKW	Schweizerischer Kosmetik und Waschmittelverband
SDB	Sabouraud dextrose broth
XTT	2,3-bis-(2-methoxy-4-nitro-5sulphenyl)-(2H)-tetrazolium-5-carboxanilide
(v v^{-1})	volume over volume
WMs	Washing machine(s)
(w v^{-1})	weight over volume
(w w^{-1})	weight over weight
YM	Universal medium for yeast

Summary

In the last two decades biofilm formation in household washing machines started to become an increasing problem for the machine itself as well as for the hygienic performance of the washing cycle. Lower washing temperatures (< 40°C) and increased use of liquid and bleach-free detergents supported the establishment of biofilms in washing machines. Although new strategies were developed to decrease or prevent biofilm formation, no test system is available to quantify the biofilm removal efficiency.

In accordance, this thesis project was aimed at the production of a single-species model biofilm that can be used for biofilm removal efficiency tests in household washing machines. It is highly important that the biofilm can be produced in a repeatable manner to obtain a standardized biofilm that enables to qualitatively and quantitatively estimate biofilm removal with staining and biochemical methods, respectively.

For this purpose household washing machines were analyzed and over 90 biofilm forming microorganisms have been isolated and identified. Three microorganisms (*Pseudomonas putida*, *Staphylococcus cohnii* subsp. *urealyticum* and *Rhodotorula mucilaginosa*) produced more biofilm compared to their reference strain from the strain collection and were considered for single-biofilm production in modified bench-top reactors.

The innovation of the reactor modification was the rotating cylinder placed on the axis to harbor the test materials (e.g. polypropylene) and to enable homogeneous mixing of the nutrients and shear forces within the reactor.

The produced biofilms were quantified and analyzed for repeatable growth with mature biofilm after 6-7 days post inoculation with 83 µg cm^{-2} protein, 197 µg cm^{-2} polysaccharide and 6.9 x 10^6 CFU cm^{-2} for *R. mucilaginosa* and with 60 µg cm^{-2} protein, 50 µg cm^{-2} polysaccharide and 1.75 x 10^7 CFU cm^{-2} for *P. putida*. The best repeatability of biofilm growth was shown for *R. mucilaginosa* on smooth polypropylene coupons.

The produced biofilms were also tested for preservation in cryo protective agents (e.g. trehalose), which enabled long-term storage of the biofilms for up to two months. The main loss of viable cells after 2-weeks of storage for *R. mucilaginosa* and *P. putida* was about one $\log_{10}$. The advantage of storing biofilm enables their

usage at a later time point. Especially, when the producers of the standardized biofilms will not be the endusers product needs first to be stored and transported to the enduser (e.g. manufacturer).

Fast and cheap methods are desired for the determination of the biofilm and its removal. Coloring experiments for the qualitative measurements revealed that crystal violet is the most appropriate staining method for determination due to the high contrast between the material and the stained biofilm.

The demand for standardized biofilms is large because every laboratory is producing its own biofilms for antimicrobial or removal tests making comparisons rather difficult. This study could also serve as basis for new standards for cleaning purposes dealing specifically with the removal of biofilms. The application of this model biofilm is not limited to household washing machines and could be extended in the medical field, cleaning and hygiene as well as in food industry.

Zusammenfassung

Die Bildung von Biofilmen in Haushaltswaschmaschinen hat in den letzten 20 Jahren stetig zugenommen. Biofilme haben nicht nur einen negativen Einfluss auf die Maschine selber, sondern auch auf ihre resultierende hygienische Waschleistung. Biofilm entsteht vermehrt durch die Benutzung von Flüssigwaschmitteln, bleichefreien Waschmitteln und nicht zuletzt durch die geringeren Waschtemperaturen (< 40°C) die dem Verbraucher nicht nur ökologische, sondern auch ökonomische Vorteile verschaffen. Obwohl neue Strategien für die Biofilmreduktion entwickelt wurden, ist zum heutigen Zeitpunkt kein quantitatives Testsystem zur Messung der Effizienz der Biofilmbeseitigung (auf dem Markt) erhältlich.

Im Rahmen dieser Dissertation sollten monospezifische Modelbiofilme produziert werden, die sich für Biofilmbeseitigungstests in Haushaltswaschmaschinen eignen.

Grundvoraussetzung ist reproduzierbare und standardisierte Biofilme herstzuellen, um einen qualitativen und quantitativen Nachweis zur dessen Beseitigung zu gewährleisten. Zu diesen Methoden zählen sowohl Färbereaktionen als auch biochemische Methoden.

Über 90 biofilmbildende Mikroorganismen wurden zu diesem Zweck aus Haushaltswaschmaschinen isoliert und analysiert. Drei der Isolate, *Pseudomonas putida*, *Staphylococcus cohnii* subsp. *urealyticum und Rhodotorula mucilaginosa* produzierten mehr Biofilm als die entsprechenden Referenzorganismen der deutschen Stammsammlung (DSMZ) und wurden für die Produktion der monospezifischen Biofilme in modifizierten Benchtop-Reaktoren getestet.

Eine innovative Reaktormodifikation wurde durch den rotierenden Zylinder, der auf der Achse des Rotors platziert wurde, etabliert. Daran wurde das Testmaterial (z. B. Polypropylen) befestigt und gewährleistete so eine homogene Durchmischung der Nährstoffe und Scherkräfte innerhalb des Reaktors.

Die produzierten Biofilme wurden quantifiziert und bezüglich Wiederholbarkeit der Biofilmbildung getestet. Ein reifer Biofilm auf glatten Polypropylencoupons bildete sich 6-7 Tagen nach der Inokulierung. Für *R. mucilaginosa* mit 83 µg cm^{-2} Protein-, 197 µg cm^{-2} Polysaccharidgehalt und 6.9 x 10^6 KBE cm^{-2} und für *P. putida* mit 60 µg cm^{-2} Protein-, 50 µg cm^{-2} Polysaccharidgehalt und 1.75 x 10^7 KBE cm^{-2}. Die beste Wiederholbarkeit der Biofilmbildung zeigte *R. mucilaginosa* auf glatten Polypropylenecoupons.

Die produzierten Biofilme wurden auch auf ihre Konservierungseigenschaft in verschiedenen kryoprotektiven Agenzien (z.B. Trehalose) bei verschiedenen Temperaturen getestet. Dies ermöglichte eine bis zu zweimonatige Lagerung, wobei die optimale

Lagerungsdauer zwei Wochen betrug. Es konnte gezeigt werden, dass nach zweiwöchiger Lagerung bei 4°C der Hauptverlust an lebenden Zellen bei nur einer $\log_{10}$-Einheit lag. Der Vorteil der Lagerung besteht darin, dass Biofilme jederzeit verwendet werden können. Insbesondere, wenn der Produzent des standardisierten Biofilms nicht der Endverbraucher ist, dann müssen die Biofilme zuerst gelagert und zum Endverbraucher transportiert werden.

Schnelle und kostengünstige Methoden sind für die Bestimmung der Biofilme und deren Beseitigung erwünscht. Färbeexperimente für die qualitative Messung zeigten, dass Kristallviolett sich am besten für die Bestimmung eignete, da es den höchsten Kontrast zwischen dem Material und dem angefärbten Biofilm lieferte.

Die Nachfrage nach standardisierten Biofilmen ist gross, da die meisten Labore ihre eigenen Biofilme für Antibiotika- or Removaltests herstellen und dies Vergleiche von Resultaten erschwert.

Diese Doktorarbeit kann als Grundlage für die Entwicklung neuer Standards dienen, die sich auf die Evaluation von (neuen) Reinigungsmethoden von Biofilmen beziehen. Die Anwendung dieses Modellbiofilms ist nicht nur auf Haushaltswaschmaschinen beschränkt, sondern kann sowohl in der Medizin, im Reinigungs- und Hygienesektor als auch in der Lebensmittelindustrie verwendet werden.

1. Introduction

The first fossil records of life on earth are 3.5 – 3.8 billions of years old and are the stromatolites (Dupraz and Visscher 2005) (Figure 1.1a and b). These fossils are not single microbes but rather part of an association or grouping of cells also called "Biofilms". Antonie van Leeuwenhoek made the first descriptions of a biofilm in the seventeenth century when he examined his dental plaque (Sklavin 1997) under the microscope (Figure 1.1c). For the last 150 years, the general assumption was that bacteria lived as unicellular, free-floating, planktonic cells and were therefore the main form studied (O'Toole and Ghannoum 2004). Hence, the era of biofilms in science just started a few decades ago and the theory of biofilms as a predominant living form of bacteria was formulated at the end of the 1970's (Donlan and Costerton 2002).

Figure 1.1a) Stromatolites, b) Cross-section of a stromatolite, c) Bacterial plaque bacteria discovered by Antonie van Leeuwenhoek.

1.1 What is a biofilm?

In the biofilm field several definitions of a biofilm exist. The general and well-accepted definition of a biofilm is that it is a "cell assembly attached onto a solid surface embedded in a matrix consisting of extracellular polymeric substances (EPS)". Some researchers expand this definition and include flocs (floating cell-cell aggregates), microbial mats and colonies growing on agar plates (Turner et al. 2008). The slimy, viscous EPS matrix is produced by the cells of the biofilm and consists of polysaccharides, proteins and nucleic acids but also of lipids, glycoproteins and humic acids (Sutherland 2001a,b; McSwain et al. 2005). Besides these compounds also inorganic material (e.g. ions) and detritus can be found (Sutherland 2001a).

The physiological and phenotypical properties of planktonic cells differ profoundly of those from sessile cells as up to 70% of the genes are differently expressed (Sauer et al. 2002).

Direct observations in different ecosystems revealed that over 99% of the bacteria are present as biofilms (Costeron et al. 1999; Donlan and Costerton, 2002). This suggests that the physiological state as biofilm has advantages for species that form them. Nevertheless, not all microbial cells living within a biofilm are capable of directly attaching to surfaces and to build up a biofilm (Cook et al. 1998). Some species evolved depending on an existing biofilm layer that has been built by primary colonizers in order to grow as secondary colonizers on top of them. Although different biofilms share several characteristics among each other, every single biofilm is a unique community (Tolker-Nielsen and Molin 2000).

1.1.1 Importance of biofilm study in human activities

In natural environments multispecies mixed biofilms (multi-bacterial or bacterial-fungal biofilms) are common while monospecies biofilms are rare (Sutherland 2001a). It has been assumed that a mixed oral/dental biofilm can comprise more than 500 bacterial strains (Whittaker 1996). Mixed biofilms have the advantage of a synergistic/mutual relationship between the different species. For example, an experimental consortium of *Burkholderia* sp. LB400 and *Pseudomonas* sp. B13(FR1) leads to complete degradation of the chlorobiphenyl compounds by sequential degradation. *Burkholderia* sp. LB400 is able to degrade the chlorobiphenyl to chlorobenzoates and Pseudomonas sp. B13(FR1) can mineralize 3-chlorobenzoate (Nielsen et al. 2000). Furthermore, mixed biofilms also enable physical protection of the cells e.g. against antibiotics (O'Connell et al. 2006). In special cases the presence of a specific strain can also inhibit the establishment of another strain (Hancock et al. 2010). This knowledge is useful for the control of pathogenic organisms.

From a clinical point of view, biofilms are also problematic as the rate of horizontal gene transfer is much higher than in planktonic state, e.g. virulence or resistance genes are more often “exchanged” (Watnick and Kolter 2000). This makes a biofilm an ideal environment for rapid evolution and creation of novel pathogens (Watnick and Kolter 2000) or leading to multi-resistant microorganisms (Hogan and Kolter 2002). However, for laboratory

purposes single-species biofilms are preferred due to facilitated cultivation and reduced amount of influential parameters.

1.1.2 Phases of biofilm formation

Five different phases can be differentiated during the development of bacterial and yeast biofilms: i) adsorption, ii) adherence/attachment, iii) microcolony formation, iv) maturation and v) dispersal (Figure 1.2a). The gene expression and consequently the proteome is constantly changing during the entire developmental cycle of biofilm formation (Sauer et al. 2002). A large part of the main knowledge of biofilm development was gained with pseudomonads such as *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* (O'Toole and Kolter 1998a,b; O'Toole et al. 2000). The understanding of these phases and their triggering mechanism is of great importance for the study of microbial adaptation to different environments and for the role that biofilms play in our everyday life.

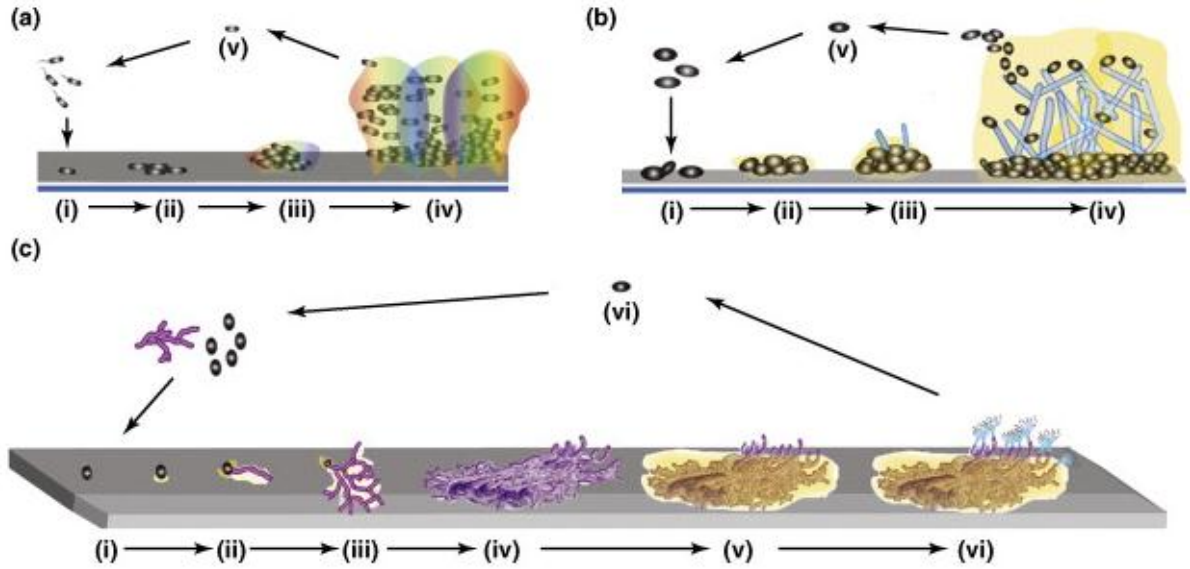

Figure 1.2. Different developmental stages of the life cycle of a) bacterial, b) yeast and c) filamentous fungal biofilms **(**Harding et al. 2009).

1.1.3 Priming and surface modification

Several studies have been conducted showing that the surface structure, topography, surface hydrophobicity or hydrophilicity plays an important role in the attachment of cells (Donlan and Costerton 2002; Reisner et al. 2003; Teughels et al. 2006).

Before cells adhere to a surface, dissolved organic compounds from the bulk fluid already adsorb to a present surface. These organic compounds produce a conditioning film. Directed surface conditioning with specific proteins (bovine serum albumin; Fletcher 1976), mussel juice (Herrera et al. 2007) or agar and gelatine have already been performed. The conditioning film can have an influence on the attachment of cells by changing the physico-chemical properties of the surface. Conditioning films with skim milk have a contra productive effect by reducing cell attachment (Parkar et al. 2003) for different Gram-positive and –negative strains (Barnes et al. 1999).

Surface structures can provide the cells with additional anchoring sites by the increase of the surface area e.g. through mechanical roughening (structures are generated with crevises) or through an adsorbed layer of organic molecules (e.g. cell debris, proteins etc.). However, the influence of surface roughness is controversial. On one hand it is believed that cracks in a surface increase the surface area for cell adhesion and giving shelter from shear forces or cleaning chemicals (Palmer 2007). In addition, increase of surface roughness results in increased surface area and therefore, there is an enhanced chance for cells to attach onto the surface. On the other hand, several scientific groups reported that an increased surface roughness does not correlate with the amount of attached cells (Vanhaecke et al. 1990; Flint et al. 1997).

The hydrophobic/hydrophilic nature of a surface can be modified to enhance cell attachment under laboratory conditions. Plasma-treatments of surfaces have become a widely used method for surface modifications. Plasma-based modification of the surface is well established and has been shown in several cases to enhance the attachment of mammalian cells under static conditions (Wan et al. 2003). The plasma generates either a thin film on the surface (thickness 30-70 nm; Hossain et al. 2007), where functional groups are embedded or the surface is directly functionalized (Gao et al. 2003). The broadly integrated functional groups are N- or O-based like amines, amid or carboxylic groups. For mammalian cell lines it has already been shown that functionalized surfaces enhance cell adherence (Girard-Lauriault et al. 2005) while for biofilm formation it has not been reported.

1.1.4 Adherence and attachment

The adherence of a cell to a surface is the phase that has been most extensively studied, because this is the first and most crucial step of biofilm development. To begin colonization on a surface the cells need the ability to overcome the forces of the surface consisting of Lifshifts-van-der-Waals or electrostatic interactions and to attach to the surface.

At the moment two theories of cell-attachment are discussed: the two-step (Marshall et al. 1971; Kumar and Anand 1998) and the three-step model (Busscher and Weerkamp 1987). In the two-step model the main stage is the reversible adhesion where rather weak interactions (Lifshifts-van der Waals, electrostatic forces and hydrophobic interaction) between the cell and the surface are taking place after the cell has been actively moving or passively been transported to the surface. Because the cells are not firmly attached, (it is a reversible adhesion process) they are still exposed to Brownian motion and therefore, they may get released from the substratum by fluid shear forces such as rinsing (Marshall et al. 1971). The second step is referred as the irreversible attachment which involves the EPS production as well as and interaction of appendages (fimbriae and pili) with the surface. It is also assumed that during the irreversible attachment an electron transfer is taking place and leading to a covalent bonding.

In the three-step model it is assumed that the main force in the initial step is the Lifshitz-van der Waals that is active at a distance of a few hundred nanometers from the substrate. In the following step also electrostatic interactions start to get involved from about 20 nm from the surface and in a last step (5 nm from the surface) adhesion receptors can enable tight adhesion of the cell.

Bacteria and yeast cells express special proteins so-called adhesins to enable or facilitate adhesion to a biotic or abiotic surface and for cell-cell recognition (Thanassi 2011). A special type of adhesins are the protruding appendages like fimbriae (Donlan 2002), type IV pili (Hori and Matsumoto 2010) or flagella (Marshall et al. 1971) that can accelerate cell adhesion and are very helpful to overcome the repulsive forces (Mafu et al. 2011). It was reported that a lack of flagella leads to problems in colonization (Sauer and Camper 2001). However, it has been shown that these appendages are not necessary for several Gram-positive and –negative strains; e.g. *P. fluorescens* is also able to attach to certain abiotic surfaces without flagella when specific nutrients are provided in the environment

(e.g. iron or citrate; O'Toole and Kolter 1998b). The necessity for appendages rather depends on the surface material, nutrient availability or shear stress than the appendages per se for cell attachment (Klausen et al. 2003).

Other investigated adhesins are the polysaccharide intercellular adhesin (PIA, a hemagglutinin) or the capsular polysaccharide adhesin (PS/A) which shares similar to identical structures as PIA) of *Staphylococcus epidermidis*. The PS/A enables the adhesion to hydrophobic surfaces (e.g. unmodified silastic catheter; Mack et al. 2009) or other biomaterials, while PIA mediates bacterial accumulation into cellular aggregates (McKenney et al. 1998).

Yeast cells bind flocculins to either proteins or sugar residues of other cells or directly to inorganic surfaces (Verstrepen and Klis 2006). In *Saccharomyces cerevisiae* specific adhesins (FLO "flocculation" proteins) play a greater role (Veelders et al. 2010) for the recognition of their own cell type. These flocculins support the formation of cells into greater flocs by recognition of sugar or amino acid residues. This ability is used in beer industry and facilitates the partition from the cells of the end product. Only one of the five different FLO proteins (FLO11) is specifically responsible for the adherence to a substrate. *Candida* species possess another type of adhesins (e.g. ALS or EPA genes) that enable the colonization of mammalian tissues (Hoyer et al. 1998; Li and Palecek 2003).

1.1.5 Microcolony formation

After adhering to the surface the cells on the surface are forming a non-organized monolayer. Moorthy and Watnick (2004) could show on a molecular level in case of *Vibrio cholerae* that the monolayer stage is a differentiated phase in biofilm development and not only a transient phase because the genetic expression of planktonic and cells in a monolayer stage is different. In case of *P. aeruginosa*, it could be shown that cells in a monolayer are able to form microcolonies by spreading over the substratum via twitching motility using their surface structures like type IV pili, while *Vibrio cholerae* uses its flagellum to move over the surface (Watnick and Kolter 1999). Another possibility to form microcolonies is the recruitment of surrounding planktonic cells (Watnik et al. 2001) or through clonal growth (Klausen et al. 2003), especially if the cell adherence is not reversible anymore. Production of daughter cells by cell division (Klausen et al. 2003) or surface migration of surface associated cells to an existing microcolony help to increase

the number of cells within the microcolonies (Watnick and Kolter 1999; Toutain et al. 2004).

In the microcolony stage about 30 - 100 cells (Kuchma et al. 2005) are closely attached to each other and build the fundament of a macrocolony (mature biofilm) with 3 - 5 layers of cells in thickness (O'Toole and Kolter 1998a). *P. aeruginosa* forms a piled 3-dimensional mushroom-type structure growing up to 70 - 100 µm under flow conditions (Reisner et al. 2003).

1.1.6 Maturation and biofilm maintenance

The maintenance of a mature biofilm (structure and other characteristics) is still not completely understood. For biofilm maintenance factors such as the quorum sensing (QS) system is activated. QS is a signalling system based on auto-induction with cell-produced signalling molecules that helps to sense the density of surrounding cells (the quorum). This system has first been discovered in *Vibrio fischeri* and *Vibrio harveyi* that are producing N-acyl homoserine lactones (AHLs) (Bassler 1993; Milton 2006). It has been demonstrated in *Burkholderia cenocepacia* H111 that biofilms of mutants of the *cepI/R* quorum sensing system were not able to overcome the microcolony phase (Huber et al. 2002).

Another maintenance factor is the general stress response system. In *Escherichia coli* the alternative sigma factor RpoS (expressed under stress conditions and also expressed in late stationary phase of planktonic cells) is playing a significant role in biofilm maturation regulating a whole set of genes (Ito et al. 2008) including cell growth (Heydorn et al. 2000) to limit the extent of biofilm formation and thus to assure nutrient availability to maintain cells viable within the biofilm.

1.1.7 Dispersal of biofilm

The last step of biofilm development is the dispersal of the biofilm. This process is less studied than the initial cell-attachment. In a clinical setting, detached or planktonic cells can initiate an acute infection or bacteremia (Costerton 1984) or lead to chronic infections (Costerton et al. 2003). Better understanding on biofilm detachment can also reduce the biofilm problem in medical or industrial sectors.

Several factors have already been proposed to play a crucial role in the detachment of biofilm.

Among these is the microbial formation of gas bubbles (Ohashi and Harada 1994), the biofilm thickness, the shear stress (Trachoo 2003), the nutrient availability (Sawyer and Hermanowicz 1998), and flow velocity (Trachoo 2003). There are different types of dispersal that can be distinguished in active and passive dispersion.

Active dispersion. The cells embedded in a biofilm have the ability to leave the biofilm by swarming and to colonize other niches (see Figure 1.2); in general this happens through dispersal of daughter cells (formation of swarming cells in bacteria, yeast spores in filamentous fungi). Factors that can trigger active dispersion are quorum sensing signals (Costerton and Stewart 2001), nutrient availability or biosurfactants.

Under stagnant flow conditions it is assumed that special quorum-sensing signals initiate cell detachment (well-studied in *P. fluorescens* (Allison et al. 1998) or *P. aeruginosa* (Boyd and Chakrabarty 1994)). Under shear conditions accumulation of QS signalling molecules is less dominant. Hunt and co-worker (Hunt et al. 2004) studied the detachment of *Pseudomonas* cells in a drip-flow reactor. Stopping the continuous flow toghether with glucose starvation triggered detachment of the biofilm. But not only limitation of certain nutrients is important but also the ratio of nutrients to each other. Rochex and Lebeault (Rochex and Lebeault 2007) made the observation that besides the concentration of C also the ratio between C : N and C : P has an impact on biofilm formation and detachment.

Genetic analysis revealed that exopolymer-degrading enzymes (lyase) are expressed around 20 h after surface colonization as function of nutrient deprivation and were missing in the earlier phase of the development (Allison et al. 1998). Biosurfactants have been described to play a role in biofilm dispersal. Biosurfactants such as rhamnolipids were mainly studied in pseudomonads (e.g. *P. aeruginosa*). During the microcolony stage they are very important for the architecture and formation of channels within the biofilm (Davey et al. 2003). However, these rhamnolipids can also inhibit biofilm development when they are overexpressed and also impair the biofilm development of other species (Davey et al. 2003). Gram-positive bacteria and yeast also produce surfactants (e.g. subtilin). In *S. epidermidis* surfactant peptides trigger biofilm maturation and biofilm detachment (Wang et al. 2011).

Passive dispersion. Spontaneous detachments of biofilm include erosion, sloughing, abrasion (Donlan 2002) or grazing (Horn et al. 2003). Sloughing, erosion and abrasion are

results from physical forces, either the shear force increased or the intermolecular/intercellular forces within a biofilm decreased (Horn et al. 2003). Erosion occurs when single cells or small clusters of cells disperse continuously from the surface of the biofilm (Stoodley et al. 2001), while sloughing mainly occurs in thick biofilms when greater parts of the biofilm are detached in a fast way (Telgmann et al. 2004). Abrasion occurs when particles collide with the biofilm (Donlan 2002).

1.1.8 The EPS matrix

The EPS matrix is the typical characteristic of most investigated microbial biofilms. The matrix surrounds the cells and fulfills several structural and protective functions; (i) it sticks the cells to each other and onto the surface giving mechanical stability; (ii) it protects the biofilm from desiccation by retention of water via hydrogen bonding, grazing, UV irradiation, heavy metals and antimicrobial agents, osmotic stress or temperature (Costerton et al. 1995; Davey and O'Toole 2000) and (iii) act as a source of nutrients (C, N, P) by storing chemical compounds in matrix which can be recycled when nutrient availability is low (Sutherland 2001b).

The matrix is not just a cluster of polymers. The components of the EPS can also interact with each other e.g. the alginate of the EPS can retain extracellular proteins (e.g lipase) preventing them washed out. This ensures that the cells can utilize the surrounding macromolecules (Flemming et al. 2007). As mentioned previously, EPS is usually produced after the irreversible attachment of the cells. Biofilm cells grown on contact lenses produced a matrix of 0.2 to 1.0 µm already 2 h after adherence (Donlan and Costerton 2002). However, some EPS have a thickness of only few nanometers (Czaczyk and Myszka 2007). In general, the amount of produced EPS highly depends on the nutrient availability in the surrounding medium: a limitation of K, N or P together with an excess of C can enhance the EPS production and additionally slow down the growth of the organism (Sutherland 2001a). Of the total EPS matrix, the main constituents besides water (up to 97%) are polysaccharides (1 - 2%), proteins (< 1 - 2%), lipids, and extracellular DNA (eDNA; < 1-2%) (Sutherland 2001b).

Exopolysaccharides are the main compound produced in a biofilm. The polysaccharides of the matrix are present in linear, branched or cyclic form (Starkey et al. 2004). Depending on the linkage and structure the matrix can rather be rigid or more gel-like. In literature, the kind of polysaccharide is further distinguished between “slime” which

comprises unbound polysaccharides and capsular exopolysaccharides which are bound to the cell membrane. The polysaccharides of the EPS are in general neutral or negatively charged in Gram-negative bacteria, whereas in the Gram-positive *S. epidermidis* they can be polycationic (Mack et al. 1996).

In some bacterial or fungal species, the polysaccahrides are not the major components of the EPS. In *Pseudomonas putida* and *Aureobasidum pullulans* the protein component is larger than the amount of exopolysacchrides (Metzger et al. 2009).

The **proteins** that have been proposed to play a biofilm-specific role are lectins and enzymes (lyase and polysaccharase). Lectins are produced by higher organisms as well as by microbes. They have the ability to reversible bind to carbohydrates mainly via hydrogen bonding and hydrophobic interactions. A function of these proteins is to enable adherence to the EPS matrix as well as to other cells within the biofilm and to facilitate metabolic interactions. The function of the lyase and polysaccharase are the control of the polysaccharide chain length and to use these polysaccharides as nutrient (Starkey et al. 2004). It has been suggested that the lyase activity could induce detachment processes as seen for several *Pseudomonas* species (Boyd and Chakrabarty 1994; Ott et al. 2001), whereas for *Streptococcus mutans* it has been noticed that polysaccharases help to modulate the biofilm structure by changing the physical and chemical characteristics of the exopolysaccharides to increase hydrophobicity (Lawman and Bleiweis 1991).

The occurrence of **eDNA** has been observed several times but was assumed to be just the result of lysed cells (Qin et al. 2007). However, several studies demonstrated that the eDNA formed a network in the biofilm that could act as an important structural component (Whitchurch et al. 2002), while other groups assumed that eDNA is mainly involved in recombination processes supporting genomic variability (Grande et al. 2011).

1.1.9 Induction of biofilm formation by quorum sensing

It is known that cells within biofilm are not just living next to each other but are also communicating with each other via small signalling molecules. A known group of molecules are the acyl-homoserine lactones (AHL) of Gram-negative bacteria. When a certain threshold of AHLs has been reached the expression of specific genes will be expressed. It has been hypothesized that QS is influencing the formation of biofilms because investigations on QS-deficient mutants of *P. aeruginosa* resulted in a biofilm that was much thinner and undifferentiated (Davies et al. 1998) and these cells adhered less

than its parent strain. It seems that the QS-systems also regulates the detachment of cells and therefore indirectly shapes the phenotype of the mature biofilm. Gram-positive bacteria possess another type of QS-system based on special peptides (Bassler 1999). In the dimorphic yeast *Candida* spp., the QS-system is based on farnesol and tyrosol that are responsible for the transition from yeast to hyphal growth and vice versa, depending on the size of the quorum (Sprague and Winans 2006).

1.1.10 Morphology and heterogeneity of a biofilm

The morphology of a single-species biofilm varies from one strain to another. There are mushroom-shaped biofilms containing water channels or carpet-like structures (e.g. *Pseudomonas*) where their formation is depending on the shear force of the liquid (Klausen et al. 2003). In addition, also filamented biofilms exist which are formed by septated cells (e.g. *Serratia marcescens*; Labbate et al. 2004). Strains with specific mutation can also show different architecture (less dense, less thick biofilms) than the wild-type strains (Kumar et al. 2009). However, the biofilm structure and morphology is not only influenced by the genetic constitution of the strain but also by external influences like substratum, nutrient availability or shear (Paramonova et al. 2007). Biofilm monolayer of *E. coli* PHL628 (curli) expressed a different morphological pattern when the substratum contained either CH_3 or NH_2 as functional groups (Ploux et al. 2007).

Biofilms are dynamic and highly heterogeneous structures, comprising gradients of oxygen, nutrients, waste products, signalling factors (Stewart and Franklin 2008), and pH (Vroom et al. 1999). The cells at the periphery of the biofilm usually encounter higher concentrations of oxygen that decreases towards the core of the biofilm that is mainly anoxic. The pH drop towards the core provides an acidic microenvironment due to accumulation of waste products (de Beer et al. 1994).

1.1.11 Tolerance and resistance mechanisms of biofilm towards biocides and antimicrobial agents

Biocides are chemical compounds that are used as antiseptics and disinfectants. They have the ability to harm and kill microorganisms in an unspecific manner acting at multiple target sites (Maillard 2002). Their effect is mostly concentration-dependent (Russel 2003) and influenced by contact time, temperature or pH (Russel 2003). Biocides contain a broad

spectrum of chemical agents ranging from phenols, alcohols, quaternary ammonium compounds (Denyer and Stewart 1998) to peroxides (de Carvalho 2007). Biocides share some similarities with antibiotics in their mode of action (Russel 2003).

Antibiotics usually have a specific target (Russel 2003) within a cell. For instance, chloramphenicol, a broad-spectrum, bacteriostatic antibiotic, blocks protein synthesis through inhibition of the peptidyltranserase. Biocides with similar mode of action are the parabens. Due to their unspecifity, parabens do not only inhibit protein synthesis but also DNA or RNA synthesis (Russel 2003).

A special feature of biofilms is the ability to cope with antimicrobial agents in a much better way than planktonic cells. This can be either due to antibiotic resistance or antibiotic tolerance.

Resistance towards an antimicrobial agent (e.g antibiotics, antiseptics, disinfectants) is mainly based on mutations of genes encoding for efflux pumps or enzymes resulting in antibiotic-degrading enzymes (Coifu and Tolker-Nielsen 2010). These genes can be easily spread within a biofilm through horizontal gene transfer. Also the production of a capsule or the expression of specific cell surface properties contribute to the resistance of a cell (LeChevallier et al. 1988).

Antimicrobial tolerance is a function of the physiological state of the cells (biofilm and planktonic). Cells not killed by the antimicrobial agent that is present in a higher concentration than the minimal inhibitory concentration (MIC) are called tolerant.

Different studies (Ceri et al. 1999; Lamfon et al. 2004) concluded that biofilms can be up to 1000 times more tolerant towards antimicrobial agents than planktonic cells depending on the tested strain (Lewis 2005) and the test agent. The increased tolerance towards antibiotics can be partially explained by the exopolymeric matrix. It has the ability to absorb the agent (Anderl et al. 2000), to reduce its concentration (Sutherland 2001a), or to retain their diffusion (Costerton et al. 1999) into the biofilm. However, not all types of antimicrobials are retained. Thus, several cationic antibiotics can be bound by the components of the matrix, while other antibiotics easily penetrate the matrix (Anderl et al. 2000). Also the growth phase and age of the biofilm cells affects the tolerance. Physiological heterogeneity within a biofilm has also been suggested to contribute to the resistance. For mixed-species biofilm it has already been shown that resistant or tolerant cells also contribute to the protections of other cells within the community (co-protection) (Stickler and Hewett 1991; Souli and Giamarellou 1998).

Further factors for increased tolerance are the age together with the resulting thickness of the biofilm (Anwar et al. 1992) and slower growth rates (Trachoo 2003). The existence of a subpopulation of small and slowly growing, non-dividing cells, called persister cells, can also explain parts of the increased tolerance. The fraction of persisters in a biofilm ranges from 0.1-10% and they are highly tolerant resistant to killing (Spoering and Lewis 2001). This cellular subtype can retrieve the cell division stage when the concentration of the antimicrobial agent is reduced. Persisters are not limited to biofilms; they have also been shown to be present in cell suspensions in the late stationary phase (Lewis 2007). This is the reason why late stationary cells can also exhibit a higher tolerance rate than exponentially growing cells.

However, this is not a general rule that biofilm cells are more tolerant or resistant towards antimicrobial agents because these properties clearly depend on the microbial strain of investigation (Olson et al. 2002; Spoering and Lewis 2001).

1.1.12 Eukaryotic biofilms

Besides bacterial strains, also several eukaryotes are able to grow as or embed in an existing biofilm. Among these eukaryotes are protozoa (e.g. *Euglena*, amoeba), algae (*Zygnemopsis* sp.), filamentous fungi (*Aspergillus* sp.), yeast-like molds (*Cryptococcus* sp.), and yeasts (*Candida* sp., *Rhodotorula* sp.). In contrast to bacterial biofilms, fungal biofilms have been neglected for many years. An exception represents *Candida albicans*, an opportunistic pathogenic yeast being a major cause of severe infections (Sellam et al. 2009). This strain was selected as a model strain for biofilm studies in medicine due to its excellent colonization capabilities on surfaces of medical devices (Kojic and Darouiche 2004). Moverover, *S. cerevisiae* has also been proposed (Reynolds and Fink 2001) and described as a yeast model biofilm former especially due to its good attachment performance onto plastics (e.g. polystyrene, polypropylene, Reynolds and Fink 2001) and stainless steel (O'Brien et al. 2007) but is clinically less relevant than *C. albicans* because it has not been found to form drug-resistant biofilms nor to produce an extracellular matrix (Beauvais 2009). As for bacterial biofilms, nutrient influences yeast attachment to surfaces. Adherence to plastics of *S. cerevisiae* was triggered under low carbon conditions but was reduced when carbon was completely lacking (Reynolds and Fink 2001). Interestingly, the ploidy of the genome also has an impact on the ability to adhere, haploid cells adhere much better than diploid cells (Reynolds and Fink 2001).

The extracellular polymers expressed by *C. albicans* biofilms are composed of 41% carbohydrates (of which are 16% glucose monomers), 5% proteins, 3% hexosamine and 0.4% phosphorous, the remaining components were undefined (Baillie and Douglas 2000). While planktonic cells produce more carbohydrates (87%) of which only 5% is glucose, more protein (8%) and less hexosamine (0.1%). The amount of phosphorous is similar (0.3%) (Baillie and Douglas 2000). It is also believed that the extracellular polymers can contribute to the resistance towards antimicrobials, however, no proof has been given supporting this assumption. However, Baillie and Douglas (2000) showed that the adherence of *C. albicans* on two different types of polyvinyl chloride surfaces led to different responses towards the antibiotic amphotericin B, a broad-spectrum antifungal agent that increases cell wall permeability (Rossomando et al. 1976). Baillie and Douglas concluded that the resistance to amphotericin B is due to highly specific, surface-induced gene expression.

S. cerevisiae tends to form flocs when lectin-like adhesins, also known as flocculins, are produced leading to enhanced cell-cell adhesion (Verstrepen and Klis 2006). For a long time it was assumed that *S. cerevisiae* does not form an extracellular matrix. Interestingly, these flocs were surrounded by a matrix that consisted of mannose and glucose. Further, the flocs were also less susceptible towards amphoterin B and ethanol. However, the authors believed that the matrix is not essential for floc formation and did not play a role for the increased resistance towards amphoterin B or ethanol. However, the role of this matrix is still not elucidated (Beauvais et al. 2009).

Biofilm formation of filamentous fungi are even less investigated because many of their developmental features do not exactly fit to the bacterial biofilm model although they are perfectly adapted to growth on surfaces. The main habitats of filamentous fungi are not expected to be aqueous systems rather than humid environments with a major air interface (Harding et al. 2009). Filamentous fungi often invade the substratum (occasionally also *Candida* species (Hausauer et al. 2005)), dimorphic yeasts, *S. cerevisiae* (Guo et al. 2000; de Nicolás-Santiago 2009). Many filamentous fungi also differ from bacteria and yeast in that they form differentiated cell structures such as invasive hyphae, structures for sporulation or host penetration (Harding et al. 2009). Therefore, the proposed biofilm definition has been adapted for filamentous fungi. According to Harding and co-workers (2009) a biofilm of a filamentous fungus is present when growth occurs in a complex manner (hyphal bundles or layers, hyphae as monolayers), on a surface and when the cells are enclosed in a secreted matrix that mainly consists of polysaccharides (e.g. based on

glucose) or polyols (e.g. glycerol in *Aspergillus* sp.; Beauvais et al. 2007) and only few protein (0.2-2% depending on the culture medium; Jeng et al. 2007)

1.1.13 Eukaryotic biofilm development

For yeast models, criteria and phenotype are available characterizing yeast biofilms. The different developmental phases of biofilm formation for yeast have been adapted from bacterial biofilms (see also Figure 1.2). Steps of biofilm formation by budding yeasts are closer to the ones by bacteria than the ones by filamentous fungi (Harding et al. 2009). Yeast biofilms share the same five phases: adsorption, adhesion, microcolony formation, maturation, and dispersal. The main difference is that *Candida albicans* and some other yeast strains are dimorphic, meaning that they start to colonize the substratum, building a thin basal layer of spherical yeast cells and producing a thicker layer consisting of pseudohyphae and hyphae (Baillie and Douglas 1999). Similar to bacterial biofilms *Candida albicans* also exhibits water channels during microcolony formation (Jabra-Rizk et al. 2004). In yeast cells, the dispersal is similar to the one displayed by bacteria. For dimorphic yeast the main form of dispersal is as blastospores (Chandra et al. 2001) rather than in the hyphal cell form. The blastospores are expressed at the end of hyphae at the top layer of the biofilm (Uppuluri et al. 2010).

A biofilm developmental model with six different growth stages was described for filamentous fungi: adsorption, active attachment, microcolony formation, maturation I (germination), maturation II and dispersal. In filamentous fungi, the maturation is mainly recognized as the phase when fruiting bodies or sporogenous cells reproducing “organs” are produced as well as aerial growth (Harding et al. 2009). In the last phase of the biofilm cycle spores or biofilm fragments are dispersed (Harding et al. 2009). Figure 1.2 depicts the different growth phases of a) bacterial, b) yeast and c) (filamentous) fungal biofilm development.

1.1.14 Significance of biofilms in industry and medicine

Biofilm formation is ubiquitous (Donlan 2002) but mainly on liquid/solid interfaces (Chole and Faddis 2003). Biofilms grow in natural and biotic as well as in inanimate, artificial environments. They are able to grow in extreme environments such as hot springs

(Reysenbach and Cady 2001) or glaciers (Stibal et al. 2006), but also in different man-made environments, e.g. industrial plants (Timke et al. 2005) or mines (Raji et al. 2008).

In industry they may have either a beneficial or negative impact on processes. Biofilms are used in different economic sectors. They are of great use in several industrial processes such as production of economically interesting metabolites (e.g. ethanol; Vega et al. 1988) or enzymes (cellulase; Villena and Gutierrez-Correa 2006), as well as for the treatment of drinking water and wastewater (Nicolella et al. 2000), degradation of industrial waste gases, toxic and environmentally harmful compounds (Singh et al. 2006) or mining by microbial leaching of copper (Olivera-Nappa et al. 2010), gold, and other precious metals (Vera et al. 2009). Biofilms in the human body (intestinal, vaginal) can also contribute to human health. Biofilms of probiotic strains can out-compete potential pathogens (Hancock et al. 2010) or produce antimicrobial molecules and suppress spreading of these organisms (Jones and Versalovic 2009).

In the clinical field mostly bacterial biofilms are a serious issue but fungal biofilms are an increasing problem (Ramage 2009). Microbial infections due to colonization of implants or indwelling catheters with bacterial (Costerton et al. 2005) and fungal (Chandra 2001) strains are relatively common examples. Especially immunocompromised individuals (cancer or HIV/AIDS patients, neonates and elderly people) are affected. Often nosocomial (hospital-acquired) infections with potential human pathogens lead to increased cases of morbidity and mortality (Wisplinghoff et al. 2004) in hospitals. The increased tolerance and/or resistance of biofilms towards antimicrobial agents additionally hinder their combating and make it more difficult to treat chronic infections (Drenkard 2003).

Biofilms cost the global industries yearly billions of dollars for control, product and energy losses (Joaquin et al. 2009). Fouling of a specific surface can lead to destruction of the material (biocorrosion; Morton and Surman 1994) and extensive biofilm formation in water or oil pipes leads to clogging (Pratt and Kolter 1999). The replacement of destroyed material is both time-consuming and extremely costly. The shipowning companies suffer from high costs triggered by biofouling. Increased resistance towards the water and therefore higher fuel consumption are consequences of fouling of the ship hulls (Brady 2001; Farrapeira et al. 2007).

In the food or paper industry the main problems are spoilage of the product or reduced quality due to decreased hygiene. Biofilm formation has been reported for domestic environments such as the kitchen (e.g. fresh products, sink, sponge or refrigerator Michaels et al. 2001), dishwasher, bathroom toilet (Pitts et al. 1998; Egert et al. 2010), shower

curtain (Kelley et al. 2004), tile walls (Hisanaga et al. 2008) or in laundry and household washing machines (Gattlen et al. 2010).

1.1.15 Removal and prevention of biofilms

Different strategies have been used to remove established biofilms. Chemical cleaning of contaminated surfaces is a way to remove biofilms but in many cases this approach is not the most effective as sole treatment. Either the chemical compounds fail to kill and disrupt the cells based on their resistance or tolerance mechanisms, or the compound kills all cells but the EPS matrix still remains on the surface of the material and could promote re-colonization by serving as anchoring sites and source of nutrients (Neu 1992; Sutherland 2001b).

A possibility to disrupt the matrix is the use of matrix-degrading enzymes (e.g. polysaccharases, polysaccharide lyase and protease) typically produced by fungi. Orgaz and co-workers (2006) tested a mixture of different fungal-derived enzymes (e.g. alginase, pectinase) on the removal of *P. fluorescens* biofilms. In contrast to this, Vickery et al. (2004) tested the cleaning efficiency of high-enzymatic detergents containing amylases and proteases to remove biofilms from endoscopes and made the observation that enzymes may partially disrupt the matrix but were not sufficiently efficient to kill the cells and remove the entire biofilm. Without mechanical stress the biofilm is not disrupted when only chemicals (Simoes et al. 2005) and/or enzymes (Oulahal et al. 2007) are applied. Therefore, usually a combination of both factors is applied during sanitation (Simoes et al. 2005). Very effective is brushing in biofilm removal (Fermandes et al. 2007). Flushing of the system can be applied if brushing is not possible (e.g. catheters; de Carvalho 2007). However, in this case a biofilm cannot really be removed completely and frequently a biofilm will quickly re-establish. Regular mechanical treatment and sanitation is necessary to keep biofilm formation at a low level (Hood and Zottola 1995).

Due to the corrosive nature of biofilms on the attached material, it would be optimal to avoid adherence of cells to surfaces. A preventive approach would be to modify the material surface to avoid microbial cell attachment.

Nanotechnology and plasma-based surface modifications are promising approaches to inhibit cell attachment. Surfaces with a self-cleaning property (lotus effect) have the ability to inhibit bacterial attachment due to their superhydrophobic nature (water contact angle > 150°). Researchers tried to mimic these effects based on carbon nanotubes treated with a

special paraffin coating that is highly water-repellent (www.physorg.com 2008; Srinivasan et al. 2008). However, the main disadvantage is that the lotus effect is not active in a wet or submerged environment (http://www.scribd.com).

Surface-functionalization through plasma-treatment can alter the surface properties either by adding a layer with an antimicrobial compound or changing the surface hydrophobicity. Thin films generated by radio frequency plasma polymerization of the organic compound terpinen-4-ol, which has a bactericidal effect, was shown to prevent microbial attachment of Gram-positive and –negative bacteria after 18 h of incubation when it was applied at 10 W. However, higher power (25 W) did not prevent cell attachment due to loss of surface functionality (Bazaka et al. 2010). Besides organic compounds also nano-composites of toxic inorganic compounds (e.g. silver with zeolith; titanium oxide) can be incorporated on a surface and the compounds are continuously released from the surface. This effect is usually limited for a few days (Samuel and Guggenbichler 2004). Enzymes such as amylases or proteases have already been developed to render cell adherence impossible or to kill attached cells (de Carvalho 2007). A further strategy would be to inhibit the cell-cell communication system with specific quorum-sensing blockers.

To effectively treat biofilms a combination of different strategies should be applied e.g. usage of enzymes to disrupt the biofilms, application of antibiotics or biocides to kill the cells and mechanical cleaning to remove the biofilms from the surface.

1.2 Laboratory-based biofilm production

Nowadays there is an abundance of different methods to produce biofilms. The biofilm models are divided into open and closed with respect to the availability of nutrients. Open systems are systems where the nutrient availability is infinite and in closed systems the nutrient availability is finite (McBain 2009). The easiest systems to handle in biofilm research are agar and microtiter plates. Chemostats are more sophisticated apparatus for continuous cultivations of biofilms (open system). The most commonly available cultivation systems in biofilm research are listed in Table 1.1.

1.2.1 Biofilm formation in closed systems

Agar plates and 96-well plates are often used to investigate short-term biofilms. **Agar plates** are a conventional method to cultivate microbial cells. However, for the cells growing on agar plates still remains disputable whether the built structures are biofilms or not. Some scientists accept the colonies formed on the agar as biofilms while Donlan and Costerton (Donlan and Costerton 2002) do not consider cells growing on agar plates as biofilms because they behave more like planktonic cells that are stranded on a solid surface. Colonies growing on an agar plate still share the features of a biofilm like high cell density and gradients of e.g. gas, metabolites or nutrients.

Agar plates can be used to cultivate microorganisms on a filter paper placed on top of the agar surface. Flat test coupons are placed on the inoculated filter paper. The cells from the filter paper form a biofilm on the coupon and can be used for antimicrobial testing (Charaf et al.1999).

A very simple way to produce young biofilms, which are up to 48 h old, is the usage of **96-well plates**. Microtiter plates are usually applied to screen a big number of biofilm forming strains simultaneously or to test the resistance/tolerance towards antimicrobials of young or established biofilms. Different applications have been already described for biofilms grown in well plates. In general the well plates are made of polystyrene because it has been shown to be suitable for a multitude of applications and also do not inhibit cell growth.

In bigger well plates (with 6, 12 or 24 wells) it is also possible to place specific test coupons into the well and submerge them with a culture with a test strain in order to measure the biofilm formed on the material.

Both systems are simple and easy to handle. A disadvantage is that in nature closed systems with finite nutrient availability are rather rare (McBain 2009).

1.2.2 Continuous cultivation of biofilm

In industrial settings mainly mature biofilms cause problems. In order to study mature biofilms an appropriate culture device is necessary. Long-term and high cell density studies can be performed in bioreactors. Depending on the question to be answered different types of reactors are used. Most of the reactors are custom-made but the basic units of reactors are usually commercially available. Reactors are more demanding with

respect to handling and require skilled operators. One of the most commonly used reactors are flow cell reactors and rotating reactor systems.

1.2.2.1 Flow cell reactors

Flow-cell reactors are one of the earliest reactor systems to study biofilm formation. Flow cells have the advantage that the development of a biofilm can be observed *in situ* with a coupled confocal microscope. Initially the supports of biofilm formation were thin-walled tubes (Korber et al. 1989) but had several limiations 1) excessive biofilm formation leads to clogging (Rundegren et al. 1992), 2) the tube has to be cut to access the biofilm, 3) the formation of a nutrient gradient (Palmer 1999). More often, modified microscope slides have to be used. This reactor type allows the researchers to analyze and quantify three-dimensional biofilm in a non-destructive manner with CLSM (Stubblefield et al. 2010) or another optical device (Figure 1.3a).

Very popular for the investigation of biofilms under a flow regime (as circulating batch or continuous culture) is the **Robbins device**. The original Robbins device consists of a brass pipe where the liquid is flowing through. Pegs are inserted into the Robbins device. The end of the peg is part of the wall of this flow reactor. The pegs can be removed during cultivation to analyze the biofilm grown on it. A modified version was already developed by McCoy and coworker (McCoy et al. 1981) for monitoring of industrial biofilm. Nickel et al. (Nickel et al. 1985) developed the more popular **modified Robbins device** (MRD) that is used for testing biofilm formation and resistance to antibiotics. The MRD is in principle an artificial multiport sampling catheter and are specialized for low flow experiments but are also adapted to high-pressure conditions (Kharazmi et al. 1999). The MRD is a practical tool when several surface materials should be tested with the same cell suspension in parallel (McBain 2009). The main application is testing industrial biofilms or biofilms from pipe systems (Figure 1.3b).

Drip–fed reactors are reactors that provide the nutrient in a dripping manner. The reactor is in an inclined position to enable the liquid to cover all the test surfaces (e.g. microscope slides). The test surfaces are not limited to glass slides, but also organic material like human tissue or sliced pork meat is used (McBain 2009). However, care has to be taken because it is possible that gradients or spatial heterogeneity of biofilm occurs due to inhomogeneous distribution of the liquid.

1.2.2.2 Rotating reactor systems

A **rotating system** has the advantage that the liquid in these systems is constantly mixed. In addition, the rotation causes a shear force on the test surface that can be controlled. Rotating systems have also been applied for testing the resistance towards antimicrobials of established biofilms under various flow conditions. Most of these reactors also allow numerous samples of biofilms at once.

The rotating disk reactor (RDR) consists of a disk placed at the bottom of the reactor vessel onto which test surfaces can be mounted. The horizontally rotating has been selected as a standard test method (ASTM E2196-07) for quantifying *P. aeruginosa* biofilms under shear flow conditions.

Another reactor type for continuous cultivation is the reactor developed by the Center of Disease Control (**CDC reactor**). The CDC reactor is a submerged substratum reactor. The test surfaces (coupons) are inserted into the reactor with special holders that are arranged in a vertical manner. Eight holders with three test coupons can be inserted. The coupons can be removed and be replaced aseptically during the cultivation period. The mixing is performed with a magnetic stirrer bar. The holders remain static in the reactor and the shear is generated by a magnetic stirrer bar. A standard protocol has already been developed (ASTM 2562-07: Standard test method for quantification of *P. aeruginosa* grown with high shear and continuous flow using a CDC biofilm reactor; http://www.biofilms.biz). This reactor has been developed to achieve high shear forces (0.06 N m^{-2}) (Goeres 2006) (Figure 1.3c).

The annular biofilm reactor consists of an inner rotating cylinder and a non-rotating outer cylinder. Slides of the test material can be inserted into the rotating cylinder of the reactor. This reactor can be applied for metal-loss biocorrosion studies or general biofilm formation. The mixing of the liquid and the regulation of the shear is done with rotation of the cylinder and controlled by a mechanical coupling to the motor on top of the reactor. Neu et al. (2001) and Huang et al. (1995) used this type of reactor for testing disinfection ability of chemical compounds (Figure 1.3d).

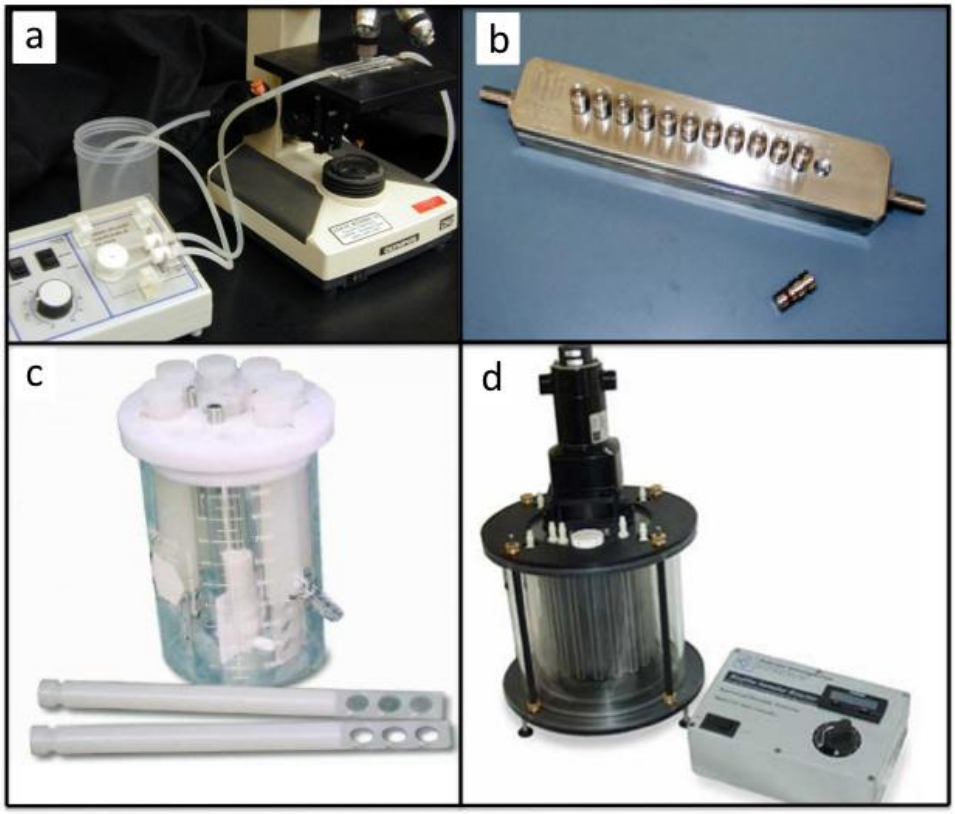

Figure 1.3. Different types of commonly used reactors for biofilm studies a) flow cell, b) modified Robbins device, c) CDC reactor and d) rotating annular reactor.

Table 1.1. Overview of commonly used methods for biofilm cultivation (McBain 2009, modified).

Cultivation system	Applications	Comments	References
Agar plate biofilm models	Basic systems for modelling biofilm, particularly those with nutrive substratum	Readily accessible and simple to run; dynamic growth (non-steady state)	(Charaf et al. 1999)
Multi-well plate biofilms	General biofilm models for replication and quantification; commonly used in biofilm molecular genetics	Simple to run, requires readily accessible materials; high degree of replication is possible; non-steady state; limited choice of substratum	(Kearns et al. 2005)
Submerged substratum models	Various; these are commonly used, general biofilm models	Representative of many real biofilm scenarios; large common fluid phase mean that replication of treatment requires multiple runs or several models; non-steady state conditions	(Bradshaw et al. 1996)
Rotating reactor	Modelling fluid flow and shear forces	Excellent for intended purpose; large common fluid phase	(Characklis et al. 1982)
The (modified) Robbins device	Modelling biofilms in flowing systems, medical biofilm, etc. A commonly used biofilm models	Readily available, robust; upstream/downstream conditions may differ	(McCoy 1981) (Nickel et al. 1985) (Kharazmi et al. 1999)
Flow cells	Real-time biofilm visualization in flowing systems etc.	One of the only choices for adhesion studies and real-time observation	(Palmer 1999)
The constant depth film fermenter (CDFF)	Dental biofilm and other general biofilm work	Simulates conditions of biofilms at the solid/air and solid/liquid interface, depending on medium flow rate; excellent for long-term continuous culture biofilm studies; biofilm depth can be set; multiple identical biofilms for time course or other replication; difficult to obtain; replication of treatments *in situ* requires multiple runs or several models	(Wilson 1999)
The drip flow biofilm reactor	General biofilm work	Simple, elegant design; biofilms may be aerially non-uniform (e.g. distribuition of biofilm)	(Buckingham-Meyer et al. 2007)
Perfused membrane biofilms	Generation of thin homogenous biofilms.	Control of growth rate, technically challenging	(Allison et al. 1999)

1.3 Optical tools for the analysis of biofilms and its quantificaiton

1.3.1 Microsopic investigations

During the early stages of biofilm research the **scanning electron microscopy** (SEM) was used. With its high-resolution capacity (ca. 5 nm; field emission scanning electron microscopy; West 2007) it could give information about the surface and biofilm structure (Singleton 1997). This technique had the disadvantage of creating artefacts because the biofilm samples that consisted of 95% water needed to be gradually dehydrated, fixed and coated. The environmental scanning electron microscope (ESEM) is based on the same principles of a conventional SEM but has the advantage to observe samples without metal coating (e.g. gold sputtering); which allows the analysis of wet samples (Donald 2003). In contrast to the conventional SEM, ESEM applies a gas (water vapor, air) and also allows the examination of samples under low pressure (1-50 Torr).

With the **confocal laser scanning microscopy** (CLSM) a new era of biofilm analysis started. Although the resolution of the CLSM is less than the one of electronic microscopes ($\geq$ 0.2 μm; Schatzlein and Cevc 1998), it enabled direct investigation of the three-dimensional biofilm architecture and the organization of the cells within the biofilm in a fully hydrated state after previous staining with fluorescent dyes. Further, the CLSM allows cross sectioning through the whole biofilm in the liquid environment in a non-destructive and non-invasive way. To receive a three-dimensional image of the biofilm it has to undergo serial sectioning by the laser scanner. The sections are made from top to bottom always at a well-defined distance. The sections are then combined with a software and result in a three-dimensional picture depicting the structure of a biofilm. The spatial distribution of the cell and EPS in a biofilm can be observed with selective fluorescent staining. Metabolically active cells could be distinguished from dead or metabolically inactive cells (persisters) with redox dye (e.g. 5-cyano-2,3-tolyl-tetrazolium chloride, CTC) that produces a fluorescent formazan crystal after being reduced by the electron transport chain reaction. Alternatively, live/dead staining can be applied where dead cells are selectively stained based on their increased permeability of their cell membrane. EPS could be made visible with lectins such as Concanavalin A or with calcofluor white. Real-time *in situ* analysis and quantification can be conducted, e.g. when the microscope is an integrative part of a reactor.

For the characterization of the surfaces **atomic force microscopy** (AFM) is a very useful tool with a high resolution (0.2 nm horizontal and 0.05 nm vertical resoltuion; West 2007).

AFM generates micrographs with a cantilever tip that interacts with the surface. The tip is oscillating and scans the surfaces. With the AFM it is also possible to study the overall structure and topography of a material's surface, to sample single cells and the macromolecules of the cells (Wright et al. 2010) or to perform force measurements to characterize the mechanical properties (e.g. work of adhesion, Young's modulus). In 1992, one of the first AFM-generated images was done with hydrated biofilm (Bremer et al. 1992). AFM has some limitations for the investigation of biofilm. Only small areas (50 x 50 μm^2) of the sampling material can be investigated. Further, the cantilever tip can only scan the cells from the top and not the sides of the cell wall (Wright et al. 2010). With AFM the topography of the biofilm can be determined in an aqueous environment as well as the roughness of the support material. The rather instable, viscous structure of the biofilm could easily be destructed during scanning especially in an aqueous environment. Therefore, biofilms were dehydrated and fixed. However, this step changes the characteristics of a natural biofilm. In contrast, biofilms of *P. putida* grown under humid air conditions and subsequently dehydrated for microscopy did not change their structure and morphology compared to biofilms grown in a liquid environment (Auerbach et al. 2000).

1.3.2 Quantification of biofilms

Colorimetric methods such as the crystal violet assay or redox assays (e.g. CTC) are semi-quantitative and relatively simple methods to measure biofilm formation.

Crystal violet (CV) is a relatively unspecific staining. It is a cationic compound that interacts with negatively charged ions that are major components of the EPS (e.g. alginate, uronic acid etc.) and of the cell membrane (e.g. LPS or phospholipids, teichoic acids; Stoodley et al. 1997). CV staining is cheap, fast and already well established in microbiology. The staining needs an incubation time of 10 - 30 min (Peeters et al. 2008) and the amount of biofilm can be determined via spectrophotometry of bound CV. For this, the CV is dissolved from the biofilm (destaining) with a solvent (e.g. ethanol, dimethylsulfoxid, acetic acid, methanol). The amount of dissolved CV is proportional to the amount of biofilm. The disadvantage is that it does not distinguish between living and dead cells and is not applicable for all microorganisms. For instance, *P. aeruginosa* failed to give reproducible results with this staining method (Peeters et al. 2008). Alternative, also unspecific dyes are fuchsin, congo red or methylene blue.

Viability of cells can be determined with redox dyes (CTC, INT, XTT). The reduced component is fluorescent and needs to be measured with a fluorescence spectrophotometer. CTC works relatively well but can also lead to an underestimation of biomass (Lund et al. 2003). These viability studies can also be applied to planktonic cells (Peeters et al. 2008).

1.4 Biofilms in the laundry industry

1.4.1 Washing behaviour and "Green thinking"

To date, the goal of every manufacturer of household washing machines is to develop a system that enables washing at low temperatures to drastically reduce the amount of energy to heat the water as well as the amount of water being used. The main awareness of the high energy-consumption started mainly with the energy crisis in the USA (1978) (Terpstra 1998). In 1981, about 1% of the total energy consumption in the USA, is due to laundering (Blaser 1984).

Until then, it was very common to launder at very high temperatures (60 - 95°C). Washing and drying in a common household could make up to 17 - 20% of the whole energy consumption (Nipkov et al. year unknown). In Europe, new washing machines are nowadays labelled with letters from A to G: "A" indicating an energy-saving washing performance while G are extremely energy consuming machines and should be exchanged. Changing to more energy-saving machines reduced the total energy consumption by 15% in the last decade (http://www.hausinfo.ch). Meanwhile washing machines are already equipped with specific washing programs that require less washing detergents, water and energy than two decades before (Figure 1.4). In Switzerland, a single washing cycle at 90°C costs 0.39 CHF/kWh, while washing at 40°C costs one third and at 30°C only one fifth of that (Figure 1.5). Washing at 30°C can reduce energy consumption by 80%. Also in other parts of Europe and in the USA, the average washing temperature continuously decreased to 40-60°C (Sheane 2000). In Europe over 60% of the laundry is washed at 40°C or less (Block and Stelter 2002). Figure 1.6 represents the trends of temperature changes over the last 20 years in Switzerland.

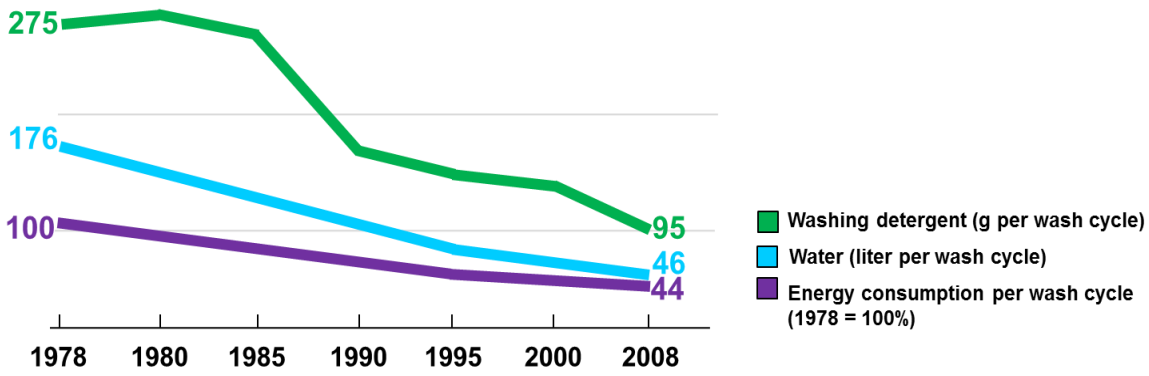

Figure 1.4. The washing behaviour changed drastically over the last 30 years. The amount of washing detergent (blue), water (in liter) and energy consumption reduced per washing cycle (Schweizerischer Kosmetik- und Waschmittelverband 2010).

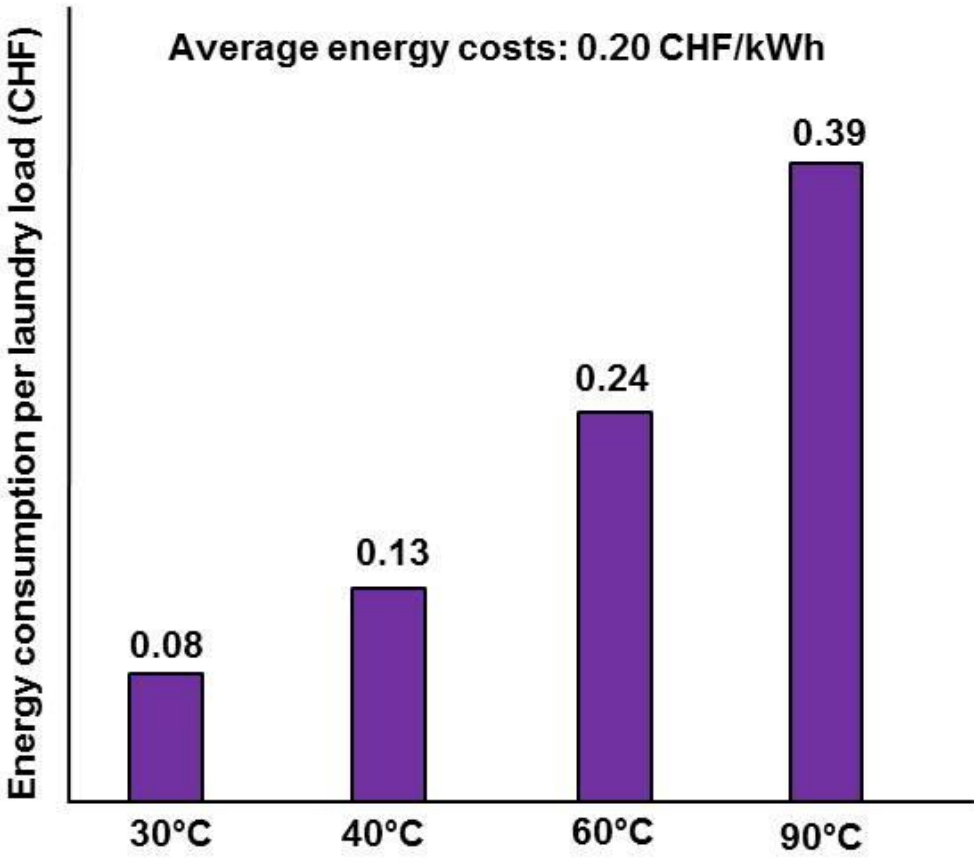

Figure 1.5. Average cost for energy consumption for the different washing temperatures (30-90°C) in Switzerland (Schweizerischer Kosmetik- und Waschmittelverband 2010, modified).

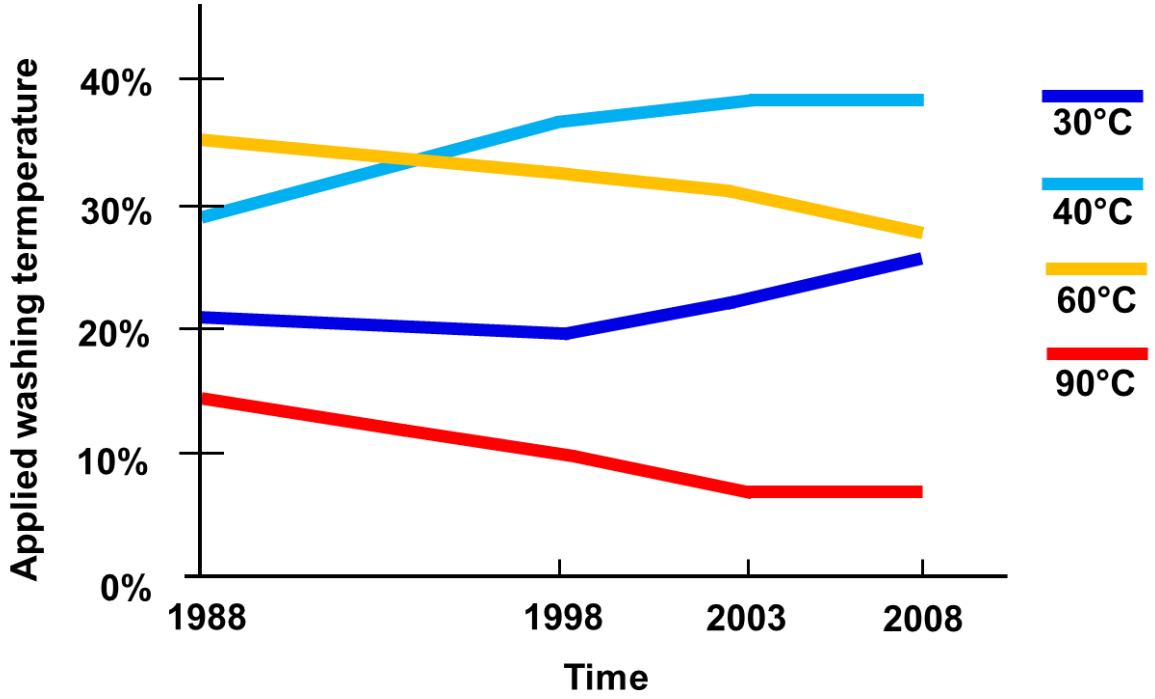

Figure 1.6. Percental usage of each washing temperature (lower panel) over the last 20 years (1988-2008) in Switzerland (Schweizerischer Kosmetik- und Waschmittelverband (SKW) 2010).

1.4.2 The detergent market

The modern washing detergents were produced first time in 1959, until then, mainly conventional soap or soap containing soda and bleaching agent, was used. The conventional soaps were replaced by tetrapropylenesulfonate, a tenside produced on petrol-chemical basis. However, this tenside triggered extreme foam production and needed to be replaced by linear alkylbenzolsulfonates that are better biologically degradable. In the middle of the 80s, phosphate-free detergents were available to reduce the overfertilization of the waters (http://www.seilnacht.com).

Today, a plethora of different types of detergents are available (heavy- and light-duty detergents, modular detergents and special detergents) ranging from powder to liquid formulations. In 1992, liquid detergents were introduced and gained much popularity. In 1999, a trend from powder towards liquid detergent has been observed in the U.S. (Teng 2000). Although most of the regular powder detergents had a very good performance, detergent producer change the composition regularly (Stiftung Warentest 2002) for marketing reasons. In general, the components of laundry detergents are always the same: tensides, bleaches, enzymes, and water softener.

The **heavy-duty detergents** are usually produced in powder form but are also available as liquid detergent. They contain bleach and can be used for washing at 20 - 90°C mainly for white textiles. Special **color detergents** (powder or liquid) do not contain bleach and are usually applied for washing at 20 - 60°C. **Light-duty detergents** also do not contain bleach at all. The main washing activity is based on enzymes that remove soils from delicate textiles.

1.4.3 Washing behaviour in Europe

The five biggest countries in Europe were investigated for their washing behaviour in terms of product usage and applied temperature (Ecolabelling Denmark 2009). In Spain, nearly 90% of the laundry is washed at temperatures lower than 40°C. In Great Britain or Germany the main washing is performed at 40°C while only 4% of the laundry is washed at lower temperatures. In all the investigated countries, powder detergents are still the most popular detergents, whereas Germany prefers the usage of compact and regular powder for a washing, followed by liquid detergents (26%) (Table 1.2).

Table 1.2. Washing behavior and usage of washing detergents in the 5 biggest European countries (Great Britain, Germany, France, Italy, and Spain) (Ecolabelling Denmark 2009, modified).

Characteristics	Detergent form	Product	Great Britain	Germany	France	Italy	Spain
% Usage of different products of total washing detergents on the market	Powder	Regular powder	49	25	38	57	66
		Compact powder	0	40	4	0	2
		Tablet	28	8	17	2	9
	Liquid	Liquid	16	26	37	41	23
	Mix	Liquitab	2	1	4	0	0
% Loads of washed at different temperatures		< 40°C	4	4	30	40	87
		40°C	69	69	36	23	9
		50°C	9	9	4	7	1
		60°C	15	15	21	24	2
		> 70°C	3	3	9	6	1

1.4.4 Washing performance

The Sinner circle, an evaluation model developed by industry (Henkel AG & Co. KGaA), shows that five parameters define a washing cycle: water, temperature, time, mechanics and chemistry. If one parameter is changed, the other parameters have to be adjusted to result in the same washing performances. With the reduction of the thermal constituent to 40°C, the washing time is shorter because the water takes less time to be heated up, the mechanical action is usually a function of the textile and therefore the chemical part needs to be increased to result in the same washing performance like at 60°C (Figure 1.7). The detergent industry was challenged to create washing detergents that are active at low temperatures and still ecologically friendly (biodegradable).

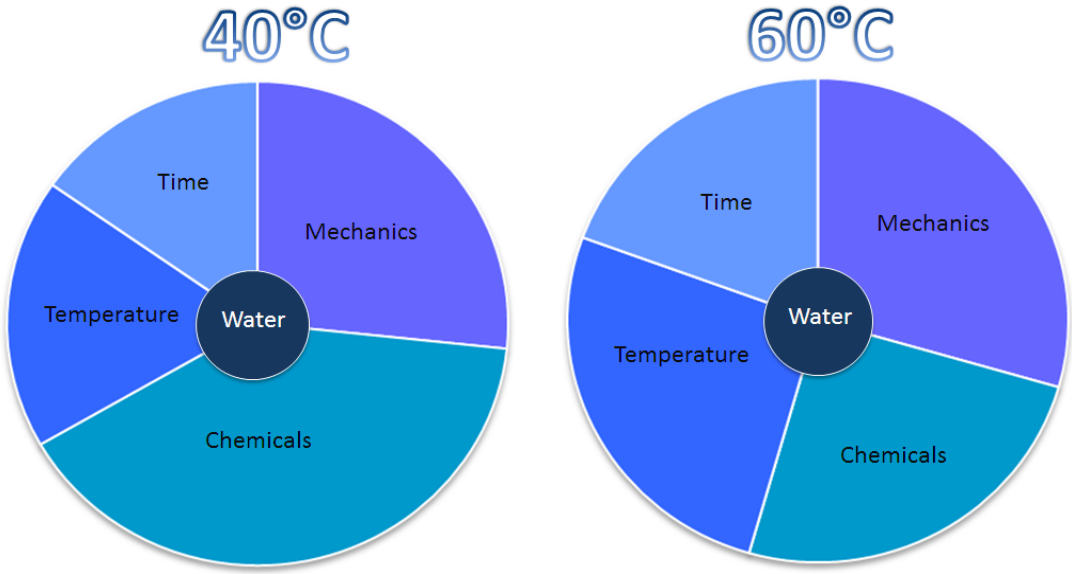

Figure 1.7. Sinner circle. Washing performance is a combination of water, temperature, time, chemistry and mechanics. When the temperature is decreased, new chemicals have to be applied to enable same quality of cleaning (Schweizerischer Kosmetik- und Waschmittelverband 2010).

1.4.5 Biofilm growth in household washing machines

The social and economic changes have a great influence on the development of biofilms in household washing machines. Numerous end users of household washing machines complained about the formation of malodour when the washing cycle was run at reduced temperatures. Also manufacturers of household washing machines made the

observation that biofilm formation occurred especially in regions with hard water where deposits of calcium carbonate and remaining washing detergent provide microbes with essential nutrition for the establishment of a biofilm (Ecolabelling Denmark 2009). The emergence of biofilms in this specific environment is a direct consequence of the changes in laundry behaviour. Washing at 60°C was usually sufficient to kill potentially pathogenic microbes. In addition, the enhanced use of liquid detergent could contribute to the biofilm formation because the washing performance is less effective than powder detergents and they lack bleach (Stiftung Warentest 2002). The microorganisms are mainly introduced into the washing machine by dirty laundry or particles of human skin (Munk et al. 2001). The bacteria that survived the washing cycle proliferate in the humid, warm environment of a washing machine. Theoretical consequences of surviving bacteria in a household washing machines are transfer of bacteria causing human infections, microbial damage of fabrics and malodour production along with intense biofilm formation (Munk et al. 2001). Established mature biofilms can regularly release cells during the laundering process, contaminate the laundry and impair the hygienic performance.

Not only the washing behaviour, but also the type of washing machine can influence the occurrence of biofilms in the machine. Front loader washing machines are reported to be more problematic than top loader. Although the washing drum is still in the vertical position, the main construction of the washing machines is different. Front loader washing machines have the tendency to accumulate the wastewater in the machine. This provides the system with humidity and remaining washing detergent, soil and dirt particles. These conditions are the best prerequisite to enhance biofilm formation in the washing machines. Deposition of carbonate can protect the biofilms from the mechanical and or chemical activities of a washing cycle. These inorganic depositions serve also as a source of nutrients.

Biofilms in the household washing machine can also be responsible for biocorrosion. In the worst case this leads to local water spill or even total destruction of the washing machine. Microbes are also able to degrade the plasticizer in the plastic parts (water pipes etc.) and use it as a carbon source (Flemming 1998; Szewzyk et al. 2000) leading to disintegration of the plastic. Therefore, regular cleaning measures need to be applied.

1.4.6 The antimicrobial action of washing detergent

Although the primary goal of the whole washing process is to remove stains and then microbes, some components of the washing detergents have biocidal activity. Due to the mechanical, thermal and chemical forces most of the microorganisms are removed during laundering.

Tensides. The major components of a washing detergent are tensides. In the washing detergent a mixture of cationic, anionic, non-ionic and amphoteric tensides are used. Their function is to reduce the surface tension of the water and therefore to enable easier removal of oily and greasy stains. In respect to bacterial contamination they also have a bactericidal effect by impairing the cell membranes. They reduce the surface tension of the cell wall, solubilize membrane-bound proteins (Denyer 1990) and make the cell membrane permeable for biocides and/or enzymes that disrupt the matrix.

Bleach. Bleach was regularly added to the washing process of predominantly white laundry to remove soils and stains from fabrics. Furthermore bleaching compounds have a bactericidal activity and are therefore also used as disinfectants of surfaces. Two types of bleaches became widely used: oxygen-based and chlorine-based (sodium hypochlorite) bleaches. The main targets of chlorine are enzymatic reactions as well as the denaturation of proteins (Rutala and Weber 1997). Oxygen-based bleaches (e.g. sodium perborate) produce hydrogen peroxide that is active against stains (Schweizerischer Kosmetik- und Waschmittelverband (SKW 2010)) and microbes (McDonnell and Russell 1999).

Below 60°C the oxygen-based bleaches are not adequate anymore they are less effective and need a certain water temperature to be activated. Oxygen-based bleaches have their highest activity at 60°C at an optimal pH of 10 - 11 (http://www.seilnacht.com). The addition of bleach-activators such as tetra acetyl ethylene diamine (TAED) enables washing also at lower washing temperatures. TAED reacts with the “per-species” of a detergent formulation such as sodium perborate leading to the production of a peracetate anion. This reaction is already activated at 30°C (SKW 2010). A bactericidal effect for several bacterial strains has been described for the peracetate anion under low-temperature laundry conditions (Sheane 2000). The usage of bleach is country-dependent. In the USA, Japan or Southern Europe the chlorine-based bleaches are very popular because they can be used at low temperatures. In Italy and Spain the main washing temperature is less than 40°C and about 60% of the textiles are washed with regular powder formulations where in 30 - 50% of the cases bleach or additives are added to the laundry (Table 1.2). The usage

of chlorine-based bleaches is rather problematic because it could form carcinogenic, organocholic compounds (AOX) together with the wastewater (http://www.seilnacht.com). In Switzerland chlorine-based bleaches are not commonly used in household laundering (SKW 2010).

Although bleach (e.g. hypochlorite) has a bactericidal activity and could therefore reduce the malodour production, it cannot be used for all types of garments without damaging the colours of the textile (Tobe et al. 2005).

Enzymes. Enzymes (proteases) have been added to the laundry detergent in the 1960's especially to replace the phosphate (Maase and van Tilburg 1983). The advantage of enzymes is they do not harm the environment and are biodegradable. However, in some cases they provoked contact allergies or asthma and are therefore encapsulated.

The biotechnologically produced enzymes are used to remove organic soils from the garments. They are present in most of the washing detergents. In delicate textiles they are the main stain remover. The enzymes belong to proteases, lipases, amylases and/or cellulases (Maps Enzyme Limited, year unknown; SKW 2005). These are components that could destroy cell structure. However, few reports are available with respect to enzymes with antimicrobial activity used for sanitation (Fuglsang et al. 1995; Hansen et al. 2003).

Innovative changes in enzyme properties via protein engineering resulted in more active, efficient and more robust enzymes in terms of pH, temperature and/or chemical stability (Procter and Gamble, year unknown). Modifications and directed evolution (Cherry and Fidantsef 2003) of interesting genes also open the spectrum of tools to find improved and more efficient enzymes.

1.4.7 Industrial laundering

In commercial industrial laundering biofilm formation in the washing machines has not been reported to be a problem. Either it has not been noticed (no malodor production, or corrosion) or the washing conditions are different compared to household washing. In the food industry and in hospitals it is of essential importance that not only the soils are removed but also that the textiles are free from germs that could be a source for spoilage or infection. Therefore, special guidelines have been developed for these sectors (Aarnisalo et al. 2006).

In food industries, protective clothing is needed to prevent direct contact of the worker with the food (e.g. in meat processing industry (Bolton et al. 2001)). In hospitals also the

employees and patients need to be protected. It is assumed that dirty laundry can act as a source for cross-contamination and could therefore provoke infections. For hospital laundering, the guidelines indicated to wash the laundry at temperatures >71°C for up to 30 min (Blaser 1984). Health care facilities also aimed at reducing the amount of consumed energy. It was considered to reduce the washing temperature because during the 1940's when the guidelines were developed, hot water active detergents were used (Blaser et al. 1984). Studies by Fijan and coworkers (Fijan et al. 2007) showed that potential human pathogens (*Enterococcus faecium*, *Staphyolococcus aureus*, *Enterobacter aerogenes*, and *P. aeruginosa*) could still survive laundering at 60°C whereas at 75°C no microbe survived. Further testing is needed for establishment of new guidelines for lower temperatures. Drying and ironing are further steps that reduce the number of microbes on the fabrics. In addition, the whole laundering process is spatially divided in a sector for dirty and for clean laundry to avoid the risk of cross-contamination (Electrolux, year unknown).

An upcoming and promising method is ozone laundering. It is water and energy saving and has shown good performance in cleaning and disinfecting fabrics. Most of the water does not need to be heated up because ozone is also active in cold water and needs fewer detergents. Ozone is more effective than other bleaching agents. A methycillin-resistant strain of *S. aureus* or *Clostridium difficile* is killed in 3-6 minutes and is a milder treatment for textiles (Rice et al. 2009). In UK ozone laundering has been applied already in the 90's for commercial laundering (Cardis et al. 2007).

1.4.8 Prevention of biofilm formation in household washing machines

As it is known that biofilms impair the hygienic performance of laundering (Terpstra 1998), different strategies and washing programs are developed that can kill the cells and also remove biofilms. The commonly known "hygienic program" (e.g. Miele.) can be applied to clean the washing machine (Bazzi 2002, EP 0808936B1).

In washing machines without these modern programs, it is generally recommended to wash the washing machine regularly with a bleach-containing detergent at 60°C to remove malodour and microbial load.

Manufacturers (e.g. Samsung) developed a system that actively releases silver ions to the inside of the machine to prevent biofilm formation. The antibiotic effect of silver was already known in the ancient world (Borsuk et al. 2007). It has also been shown to affect

several yeasts strains, fungi and viruses (Gong et al. 2007) and are commonly applied in cosmetics (Silver 2003), textile (Rai et al. 2009) or medical industry (Chen and Schluesener 2008). The bactericidal effect of silver ions on planktonic cells is due to the inactivation of enzymes by binding to thiol (—SH) groups. Further the silver ions bind to the cell wall but also on to nucleic acids (Silvestry-Rodriguez et al. 2008). Constant release of silver should prevent biofilm formation by killing the microbes (bacteria, fungi and viruses; Gong et al. 2007; Radzig et al. 2009)). However, the antimicrobial effect and way of action of silver ions and silver compounds is still controversial. Bjarnsholt and colleagues (Bjarnsholt et al. 2007) showed that the effect of silver compounds on *P. aeruginosa* is concentration-dependent. Further, they demonstrated that mutants with an impaired quorum-sensing system were more sensitive towards antimicrobial agents. In the environment several strains exist that are resistant towards silver and the increased use of silver can select for resistant microbes (Silver 2003).

Some manufacturers use the steam cleaning strategy to sanitize the laundry (LG) and/or the washing machine itself (V-Zug). According to the British Allergy Foundation, steam can reduce the amount of allergens in the laundry. Also an ultrasound washer has been introduced to the Japanese market (Sanyo) but was not very successful due to poor cleaning performance (http://business.highbeam.com; http://www.rolf-keppler.de).

A search in the European and American patent database revealed that the biofilm issue in household washing machines is a well-known problem. Improved or new mechanisms have been proposed to decrease the formation of biofilms and to clean the washing machines in the inside:

A washing machine cleaning system had been suggested that reduces the impurities on the outer washtub of the washing machine and the laundry in an automatic manner (Caetano et al. 2010, US 20100089100). Another strategy is to sanitize the outer washing machine parts with a combination of i) a halogene-based oxidizing compound that avoids corrosion of the metal alloy parts (in comparison to regular washing with hypochlorite) ii) an alkaline builder (based on silicate, phosphates or carbonates) and iii) a halogene-stable surfactant (van Buskirk et al. 2009, US 7517413).

Johansen and Munk patented a method where the malodor problem triggered by biofilms could be reduced by usage of enzymatic reactions (Johansen and Munk 2002, US 20020178509A1). The addition of lysostaphin to the washing detergent decreased the number of viable cells and resulted in a delay of cell recovery on swatches (textile samples). Bettiol and co-workers (Bettiol et al. 2002, US 6465410) found another

enzymatic additive that exhibited a good washing performance on protein-based stains and had an antimicrobial activity.

1.5 Application of a standardized biofilm model

In most settings (clinical, industrial, private) the presence of biofilms is not desired due to impairment of product quality, material damage or as potential source for pathogenic infections. Thus, prevention, control and removal of these biofilms is usually attempted with chemical (e.g. antimicrobial agents) and/or physical methods (e.g. elevated temperature, scraping). However, no test method exists that could determine the anti-biofilm performance in a reliable manner and to justify its efficiency.

A simple way to test the anti-biofilm effect of a process is to produce a standardized model biofilm that serves as reference. After exposure of the model biofilm towards a certain treatment, subsequent analysis of the remaining biofilm will give information on the adverse effects, facilitating the qualitative and quantitative evaluation of the treatment (in comparison to the untreated reference).

Models in general only depict a simplified version of the reality, only fulfilling as much of the requirement as needed. Therefore, the requirements of a model vary from one specific case to another.

For example, biofilms grown in 96-well plates have established as model biofilm for testing, e.g. action of specific active agents such as antimicrobials upon biofilms (Amorena et al.1999; Nishimura et al. 2006).

Model biofilms grown in 96-well plates are good indicators for anti-biofilm treatments. However, the biofilms are limited to 24-48 h old biofilms due to nutrition deprivation. Quantification of biomass (matrix and cells) and viability assays are performed using crystal violet stains and tetrazolium salts, respectively.

The main disadvantages of the 96-well-plates for biofilm formation are that growth is only possible on polystyrene surface and that these model biofilms are not applicable for *in situ* experiments (real use conditions).

On the other larger wells (6-24 wells) enable placement of pre-cut material (coupons) into the well, so that biofilm formation is not restricted to the polystyrene surface of the plate and makes *in situ* experiments possible.

However, biofilms grown in well plates are considered highly artificial and simplified because the conditions in well-plates hardly represent an environmental setting.

Biofilm reactors are also artificial but more sophisticated systems than well plates attempting to stimulate biofilm formation. Several parameters such as nutrients, shear force can be controlled, approaching "natural" conditions and therefore, resulting in an increase of the value of the model biofilm. Further, reactors usually use material coupons as support for biofilm formation. This enables the implementation of the biofilm within the system of investigation for application of real use conditions (e.g. testing removal efficiency of household washing machines or washing detergents within the machine).

Because biofilms can be retrieved from the reactor at any time point of their development, the effect of anti-biofilm measures towards young and mature biofilm can also be assessed.

Model biofilms are of great interest for industrial and clinical application due to the plethora of settings, wherein biofilm is a dominant problem.

The chemical and pharmaceutical industry investigating anti-biofilm agents could profit from the model biofilm for examining new compounds or target-oriented improvement of existing antibiotics, pestizides or detergents.

Further, model biofilm could serve as basis for revisions of existing or developments of new standards testing the susceptibility of microorgansims towards antimicrobials. Another interesting branch is the food industry, where biofilm models could be applied for the determination of cleaning-in-place systems. The usage of the biofilms as biosensors indicating the presence of toxic compounds is also conceivable. Chapter 7 will discuss some of the applications in more detail.

Although the development of an appropriate model biofilm together with the appropriate analytical methods (e.g. quantification method, microscopy) is challenging, it represents a promising and powerful tool to analyse and to improve anti-biofilm strategies.

1.6 Aim and structure of the Ph.D. thesis

The aim of the presented thesis is to produce a standardized biofilm with washing machine isolates in a modified bench-top reactor that could be applied to test washing efficiency of household washing machines. The effect of biofilm removal is assessed with the CV staining and microbiological and biochemical methods. The model biofilm is an integrative part of a kit to test biofilm removal efficiency.

To approach this goal, the identification of microbes growing in washing machines is necessary to get an insight of the microbial composition and finding the most representative candidate strains covering bacteria and yeast in order to produce single-species model biofilms. Biofilms produced with washing machine strain seem to be better alternatives to reference strains (from culture collections) because they are expected to be adapted to the washing machine environment. These adaptations are crucial for the survival of biofilms in the washing machine environment that exhibits chemical, thermal and mechanic stresses. Comparative studies with reference strains and the washing machine isolates are conducted to determine differences in biomass formation. Moreover, test using a standard detergent at different concentrations are performed to determine the tolerance and the behavior of the Gram-negative washing machine isolate *P. putida* and its reference **(Chapter 2)**.

Bench-top fermenters are usually used for different types of bioprocesses, e.g. high-density cultivation or analysis of PHA production. Technical modifications on the reactor enable the production of a large number of biofilms growing on pre-cut material surfaces (coupons). In the presented study continuous cultivation allows longer cultivation periods and the biofilm undergoes the different developmental phases from attachment to dispersal. The production of a single-species model biofilm with the yeast *R. mucilaginosa* **(Chapter 3)** and the Gram-negative bacterium *P. putida* **(Chapter 4)**, both recovered from household washing machines, are performed to determine the ideal cultivation conditions (e.g. cultivation period, surface material, material modifications). For the establishment of a model biofilm, it is highly important that the process of biofilm production is repeatable. To test this, four to five experiments under a specific set-up are compared. The biofilm quantification is based on optical density, the amount of proteins, polysaccharides and viable cell counts. The position of the medium inlet and sampling of biofilms are highly influential on biofilm formation and spatial variability.

Planktonic cells are usually stored under humid conditions at 4°C in refrigerators or in presence of cryo-protectants at -80°C for long-term storage. However, storage and particularly, long-term storage of biofilms is still very poorly studied. Therefore, storage of model biofilm of *P. putida* and *R. mucilaginosa* under different storage conditions (temperature and cryoprotective agents) maintaining viability, architectural stability and composition of matrix components (protein and polysaccharides) are determined. The main focus is prolongation of the shelf life of biofilms. This increases the experimental and

temporal independence for the experimenter who is not forced to use the biofilms right after cultivation **(Chapter 5)**.

When the model biofilms are produced they can be used for *in situ* washing machine removal efficiency test. The main application of the produced model biofilm and the approaches for the semi-quantitative and quantitative determination of biofilm removal in household washing machines is highlighted in **Chapter 6**.

The reactor system and procedure of biofilm production and semi-quantitative and quantitative analysis resulted in a patent (Swiss patent No. 04057/09) entitled "Standardized production of mature biofilms and methods and devices for assessing biofilm removal efficiency".

Chapter 7 gives an overall conclusion over the entire work as well as showing the future directions and potentials of the application of standardized model biofilms.

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2. Microorganisms colonizing household washing machines: *In vitro* studies of their occurance, cultivation and their behavior towards surface modifications, a standard detergent and its components

The IEC-A base detergents used in the described experiments is also referred as IEC-A* base detergent (WFK Testgewebe GmbH).

2.1 Biofilms isolated from washing machines from three continents and their tolerance to a standard detergent

Jasmin Gattlen[a], Caroline Amberg[b], Manfred Zinn[a]* and Laurie Mauclaire[a]

[a]Empa, Swiss Federal Laboratories for Materials Science and Technology, Laboratory for Biomaterials, Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland;
[b]Empa Testmaterials AG (ETM), Mövenstrasse 12, CH-9015 St. Gallen, Switzerland

*Corresponding author.
Mailing address:
Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland.
Telephone: +41 71 274 76 98
Fax: +41 71 274 77 88
E-mail: manfred.zinn@empa.ch

This chapter has been published:

Gattlen J, Amberg C, Zinn M, Mauclaire L. 2010. Biofilms isolated from washing machines coming from three continents and their tolerance to a standard detergent. Biofouling 26(8):873-882.

My contribution was the cultivation of the biofilm, comparative study between washing machine isolates and reference strains, the detergent assay and the writing of the manuscript.

Abstract

The goal of this comparative study was to investigate biofilm forming microorganisms living in washing machines (WMs). Biofilms were sampled from 11 washing machines from four countries and three continents. Among the 94 isolated strains, 30% were potential human pathogens. Representative strains were selected and biofilm formation was evaluated with the crystal violet (CV) assay. The majority of the WM isolates formed more biofilm than their reference strains. Biofilms of *P. putida* WM (the largest biofilm producer) were exposed to different concentrations (0.0007-7 g L^{-1}) of the standard detergent IEC-A* at 30°C for 30 min and observed with confocal laser scanning microscopy. Using quantitative CV assays, *P. putida* WM biofilm removal required higher detergent concentrations than the type strain. However, for both strains the recommended detergent concentration (7 g L^{-1}) was insufficient to completely clean surfaces from cell debris and exopolymeric substances.

Keywords: biofilm; tolerance; detergents; household; crystal violet; cleaning

2.1.1 Introduction

Biofilms are not only an issue in the medical field or in the food industry, they have also been found to inhabit surfaces of sanitary installations such as toilet bowls (Pitts et al. 1998), showerheads (Feazel et al. 2009) or household devices like refrigerators (Michaels et al. 2001) and washing machines (Terpstra 1998; Weide and Heinzel 2000). In washing machines, microbes are introduced by dirty laundry (soil, debris of human skin) or by insufficiently treated water. The biofilm formed in the washing machines has not been reported to be health-threatening but it is responsible for malodour (Munk et al. 2001).
Biofilms are more tolerant to chemicals and therefore more complicated to control and to eliminate than planktonic cells (Costerton et al. 1987; Stewart 1996; Simoes et al. 2006). Moreover, standard tests on planktonic cells overestimate detergent efficiency in comparison to the real conditions in a washing machine (Block and Stelter 2002). Despite this knowledge, standard tests for bactericidal activity of detergents, disinfectants and antiseptics are still conducted with planktonic cells (e.g. European Committee for Standardisation 1997a,b; ASTM International 2004). Another limitation of the standard tests mentioned above is the representativeness because test microorganisms are clinical strains such as *P.aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 6538, *Klebsiella pneumoniae* ATCC 4352 or *Candida albicans* ATCC 10231, which rarely colonize household devices (McBain et al. 2003; Feazel et al. 2009). The motivation for this study was to improve knowledge on microorganisms colonizing washing machines and to define an appropriate method to determine the efficacy of washing detergents on biofilm removal. In this study microorganisms growing as biofilms in household washing machines were first identified, as well as the hot spots of biofilm formation. In a second step, the ability of washing machine isolates to form biofilms was evaluated and compared with that of their type strain. In a third step, the biofilm tolerance against detergents was determined by estimating the detergent concentrations that were necessary to remove the biofilm.

2.1.2 Materials and methods

2.1.2.1 Sample isolation

If not otherwise mentioned all chemicals were provided by Sigma-Aldrich/Fluka, Buchs, Switzerland. Microorganisms were isolated from 11 household washing machines

and washing machine parts coming from four countries, the USA (n = 3), Switzerland (n = 7), South Korea (n = 1 and washing machine parts) and Germany (washing machine parts). Three of the washing machines were top loading the others front loading. The sampled washing machines were selected by manufacturers as relevant for biofilm investigations because, for example, they had been in use for several years or had malodour problems and eventually showed technical problems due to deposition of carbonate and detergent. The washing machines were opened and key locations for biofilm formation were visualized after staining the washing machine parts with crystal violet. Fifteen locations, such as the detergent drawer, the crossbar, the pump, the filter, the rubber ring of the door, the drum inside, the drum outside, the outer drum, the hose outlet, and the hose drum-pump were sampled with a sterile medical cotton swab (Food and Agricultural Products Standards Committee 1997). Briefly, a 2 cm x 2 cm surface was first sampled with the wet swab (sterile 0.9% NaCl solution) and then scraped again with a dry swab. Both swabs were transferred into 5 mL of 0.9% NaCl solution, vortexed for 1 min and kept overnight at room temperature to reactivate slowly growing biofilm cells. The swabs were vortexed again for 1 min followed by a 10-fold dilution series in 0.9% NaCl and plating on tryptic soy agar (TSA, Difco™, Le Pont de Claix, France) or Sabouraud dextrose agar (SDA, Oxoid, Pratteln, Switzerland). The plates were incubated at 30°C for 24 h and 48 h, respectively. Pure cultures of the isolates were established and cultured on TSA or SDA. Cryogenic stocks were prepared with overnight culture frozen in 30% sterile glycerol (1:1).

2.1.2.2 Strain identification

Gram-staining was applied followed by estimation of oxidase (oxidase reagent, bioMérieux, Lyon, France) and catalase activity (Bactident® catalase, Merck, Darmstadt, Germany), lactose fermentation (Mac Conkey agar, Oxoid) and the haemolytic pattern (Blood agar, Merck, Darmstadt, Germany). The cell size, form and the presence of endospores were determined by microscopy. Biochemical identification was conducted with API® tests (API 20 NE, REF 20050 identification system for non-fastidious Gram-negative rods; API 20 E, REF 20100/ 20160 identification system for Enterobacteriaceae and other non-fastidious Gram-negative rods; API 20 C AUX, REF 20210 yeast identification system, bioMérieux). When biochemical analysis led to ambiguous identification, strains were sent to BaseClear (Leiden, Netherlands) for sequencing and identification. For the initial samples 16S rDNA was sequenced with following primers:

16SR TACCTTGTTACGACTTCGTCCCA, 16SF AGTTTGATCCTGGCTCAG, 16S AGAGTTTGATCCTGGCTCAG, 16S ACGGCTACCTTGTTACGACTT and for further sequencing and identification the validated MicroSEQ® systems (16S rDNA (bacteria) or D2-LSU rDNA (fungi)) from Applied Biosystems (Nieuwerkerk, Netherlands) were used.

2.1.2.3 Biofilm formation and quantification

Biofilm formation was quantified for the microorganisms isolated from at least two countries that could easily be cultivated (i.e. able to grow in defined medium and not flocculating). In total 15 isolates were screened belonging to Gram-negative, Gram-positive bacteria and yeast. The washing machine (WM) isolates were compared with their type strains from the German strain collection (DSMZ) that served as reference (Table 2.1). The microorganisms were transferred from frozen stock into 5 mL of the appropriate medium and streaked on agar plates of the corresponding medium: (i) TS medium: Tryptic soy broth and agar, (ii) SD medium: Sabouraud dextrose broth and Sabouraud 4% glucose agar, (iii) Trypticase soy yeast extract medium (DSMZ medium 92): 30 g L^{-1} TS broth, 3 g L^{-1} yeast extract, 15 g L^{-1} agar, (iv) Gym *Streptomyces* medium (DSMZ medium 65): 4 g L^{-1} glucose, 4 g L^{-1} yeast extract, 10 g L^{-1} malt extract, 2 g L^{-1} $CaCO_3$, 12 g L^{-1} agar, (v) Universal medium for yeast (DSMZ medium 186): 3 g L^{-1} yeast extract, 3 g L^{-1} malt extract (Oxoid), 5 g L^{-1} peptone from soybeans (peptone N-Z-Soy BL 7, enzymatic hydrolysate), 10 g L^{-1} glucose, 15 g L^{-1} agar. A single colony was picked from an agar plate, inoculated into 15 ml of the appropriate medium and incubated (30°C, 150 rpm, Infors HT, Bottmingen, Switzerland) for ca. 15 - 18 h. Five ml of the overnight culture were centrifuged (10,000 x *g*, 4°C, 15 min, Heraeus® Multifuge® 3 S-R, Thermo Fisher Scientific, Zurich, Switzerland). The pellet was resuspended in 10 mL of 0.9% NaCl. Aliquots were taken for cell counting by flow cytometry (CyFlow®, Partec, Münster, Germany) after staining with Syto 9 (final concentration: 0.5 µM, Molecular probes®, Invitrogen, Lucerne, Switzerland). The remaining cells were stored at 4°C, centrifuged and resuspended in sterile biofilm medium to a final cell number of 10^5-10^6 cells mL^{-1}. Biofilm minimal medium (pH = 7) consisted of 1 g L^{-1} 3-(*N*-morpholino)propanesulfonic acid (MOPS), 1.1 g L^{-1} $(NH_4)_2SO_4$, 0.15 g L^{-1} KH_2PO_4, 0.25 g L^{-1} $MgSO_4$ x $7H_2O$, 0.1 g L^{-1} $FeSO_4$ x $7H_2O$, 0.2 g L^{-1} Na_2-EDTA x $2H_2O$. Autoclaved medium was supplemented with 1 g L^{-1} heat-sterilized D(+)-glucose as carbon source and 1 mL of filter-sterilized (0.22 µm, Millex®, Milipore™ AG, Zug, Switzerland) trace element stock solution (1.5 g L^{-1} $CaCl_2$

x $2H_2O$, 3.96 g L^{-1} $MnCl_2$ x $4H_2O$, 5.62 g L^{-1} $CoSO_4$ x $7H_2O$, 0.34 g L^{-1} $CuCl_2$ x $2H_2O$, 1 g L^{-1} $ZnSO_4$ x $7H_2O$, 1 g L^{-1} MoO_4Na_2 x $2H_2O$, pH = 1). The optical density was measured at 600 nm (Spectronic® Genesys™ 6, UV-visible spectrophotometer, Thermo Electron Schweiz AG, Allschwil, Switzerland) and the cell abundance was checked by 10-fold dilution series and plating. The suspension (0.2 mL) was loaded into sterile 96-well plates (TPP92096, flat bottom, Trasadingen, Switzerland). Care was taken that the same lot number of the microtiter plate was always used (Lot Nr. 20080234). The plates were sealed with adhesive tape. The cells were spun down to the bottom of the well by centrifugation (2260 x *g*, 7 min, 4°C) and then incubated (24 h, 30°C, 50 rpm, Lab-Therm LT-W, Kühner AG, Birsfelden, Switzerland). After 24 h, the suspension was discarded and the biofilm formed was washed three times (3 x 300 µL sterile-filtered tap water) and dried in a laminar sterile bench. Crystal violet (0.1% CV w v^{-1} in MilliQ® water) was used to stain the biofilm at room temperature for 30 min. The biofilm was washed five times (5 x 300 µL sterile-filtered tap water) and dried again. To destain the biofilm, 200 µL of dimethylsulfoxide (DMSO) (Huber et al. 2002) were added (30°C, 50 rpm, 2 h) and 100 µL were transferred into sterile 96-well plates (Nunc, Cat. Nr 260836, Denmark) for the measurement of absorbance at 595 nm (Varian Cary 50® MPR microplate reader coupled to a Varian Cary 50® Bio UV/visible spectrophotometer, Varian AG, Steinhausen, Switzerland). The biofilm formation experiments were conducted with three colonies (clones) on triplicate 96-well plates. Each well was considered as an independent measurement (n = 378). The OD values were used as measured. The average OD value and the 95% confidence level were calculated. One-way ANOVA (α = 0.05) was used to compare the results.

Table 2.1. Strains (WM isolates and type strains) used for biofilm screening in 96-well microtiter plates and their corresponding medium for optimal growth of the pre-cultures (Atlas 1995, 1996). a) TS: Tryptic soy medium, b) SD: Sabouraud dextrose medium, c) TSY: Trypticase soy yeast extract medium, d) GSM: Gym streptomyces medium, e) YM: Universal medium for yeast.

Strain isolated from washing machines	Medium	Type strains	Medium
P. putida WM	TS [a]	*P. putida* (DSMZ 50026)	TS
P. fluorescens WM	TS	*P. fluorescens* (DSMZ 6147)	TS
Citrobacter freundii WM	TS	*Citrobacter freundii* (DSMZ 30039)	TS
Microbacterium oxydans WM	TS	*Microbacterium oxydans* (DSMZ 20578)	TS
Microbacterium aurum WM	TS	*Microbacterium aurum* (DSMZ 20028)	TSY [c]
Microbacterium sp. WM 1	TS	*Microbacterium* sp. (DSMZ 8600)	TSY
Microbacterium sp. WM 2	TS		
Microbacterium sp. WM 3	TS		
Staphylococcus cohnii subsp. *urealyticum* WM	TS	*Staphylococcus cohnii* subsp. *urealyticum* (DSMZ 6718)	TS
Cellulosimicrobium sp. WM	TS	*Cellulosimicrobium cellulans* (DSMZ 43879)	GSM [d]
Rhodotorula mucilaginosa WM 1	SD [b]	*Rhodotorula mucilaginosa* (DSMZ 70403)	YM [e]
Rhodotorula mucilaginosa WM 2	SD		
Rhodotorula mucilaginosa WM 3	SD		
Rhodotorula minuta WM	SD	*Rhodotorula minuta* (DSMZ 3016)	YM
Rhodotorula slooffiae WM	SD		

2.1.2.4 Washing detergent assay against biofilm in 96-well plates

The efficacy on biofilm removal by the standard washing detergent IEC-A* was assessed for *Pseudomonas putida* WM and its type strain. The washing detergent was tested on 1-day-old biofilms of *P. putida* produced as described above. The washing detergent IEC-A* (5.39 g L^{-1} IEC-A base (IEC/SC 59D Home laundry appliances 2010)), 1.4 g L^{-1} Na-perborate, 0.21 g L^{-1} tetra acetyl ethylene diamine (TAED, bleach activator; IEC/SC 59D Home laundry appliances 2010; provider Empa Testmaterials, St. Gallen, Switzerland) was dissolved in cold tap water (water hardness 16.02° fH = 9° dH) and constituted the fresh stock solution. To obtain different concentrations, the stock solution was diluted with cold tap water in 10-fold dilution steps (0.0007 - 7 g L^{-1}). The detergent (200 µL) was added to each well. The plates were incubated (30°C, 50 rpm, 30 min). The wells were rinsed five times and air dried in a laminar flow bench. Removal of biofilm was evaluated by CV staining according to the protocol already described. The experiments were conducted with three colonies (clones) on triplicate plates. Each well was considered as an independent measurement (n = 72 to 108 depending on the tested concentrations).

The average values of the blanks (detergent without biofilm, *n* = 99) were subtracted from the measured values. The value obtained was standardized using the average of the negative controls (biofilm rinsed with tap water; n = 108) and the 95% confidence level was calculated.

2.1.2.5 Confocal laser scanning microscopy (CLSM)

Biofilms of *P. putida* isolated from the washing machines and its type strain were cultivated in 6-well plates (Costar 3516, Corning Inc., NY, USA) and exposed to IEC-A* as described above. Exopolymeric substances (EPS) were stained with the lectin Concanavalin-Alexa633 (final concentration 0.1 mg mL^{-1}, Molecular probes; Invitrogen) and DNA was stained with Syto BC (final concentration 0.5 μM, Molecular probes, Invitrogen) for at least 30 min (Neu et al. 2001). The samples were analysed with a confocal laser scanning microscope (CLSM, Axioplan 2 Imaging LSM 510, Zeiss). Alexa633 and Syto BC were excited at 632 and 488 nm, respectively. Images were recorded and treated with the software Zeiss LSM Image Examiner (version 4.0.0.241).

2.1.3 Results

2.1.3.1 Biofilm formation in household washing machines

Eleven washing machines were dismantled and various parts were sampled to identify hot spots of biofilm formation. Biofilm was formed on different materials within the washing machines, in particular on metal, rubber and polypropylene (Table 2.2). Corrosion could be seen in some cases on the crossbar and some calcium carbonate precipitate in the outer drum. Depending on the shipping conditions of the washing machines some biofilms were already dry while the rest still remained humid. The microbial population differed from machine to machine whereas the microbial load (plate counts) was in the same range in all the washing machines. Biofilms were abundant at places permanently in contact with water (e.g. the evacuation tube) that were hidden and not easily accessible for maintenance cleaning. Locations with increased biofilm formation were the plastic filter, metal parts of the outer drum, and rubber tubes (Figure 2.1a-e). In the inner drum, where the washing cycle takes place, no biofilm formation was observed (Figure 2.1f). Table 2.2 summarizes the 94 microorganisms that were isolated and identified. They belonged to mesophilic

(30°C), fast growing bacteria. Thirty percent among them were potential human pathogens (e.g. *Pseudomonas aeruginosa, Citrobacter freundii* and *Serratia marcescens*).

Table 2.2. Overview of isolated strains and their location within the washing machines. a) n.c.: not classified, b) E: endospore-formation, c) B: biochemical; G: genetic, d) d: dry; w: wet, e) M: metal; P: plastic; R: rubber.

Organism	**Risk group** [a] **Endospore** [b]	**Identification**[c]	**Humidity** [d]	**Material** [e]
Gram-negative strains				
Acinetobacter sp.	1 and 2	G	w	P
Bacteroides bacterium	1 and 2	G	d	M
Brachybacterium sp.	1	G	d	P
Brevundimonas diminuta	2	G	w	P
Brevundimonas vescularis	1	B	d	P
Brevundimonas sp. / *Caulobacter* sp.	1 and 2	B	d	P
Burkholderia cepacia	2	B	d	M
Caulobacter vibrioides	1	G	w	M
Chryseobacterium indologenes	2	B	w	R / P
Chrysobacterium meningsepticum	2	B	d	P
Chryseobacterium sp.	1 and 2	G	w	R
Chryseomonas luteola	2	B	w / d	R / M
Citrobacter braakii	2	B	w	P
Citrobacter freundii	2	B	w	M
Comamonas acidovorans	n. d.	B	w	P
Ensifer sp. / *Sinorhizobium* sp.	n. d., 1	G	d	M
Enterobacter cloacae	2	B	d	M
Kaista sp.	1	G	d	M
Klebsiella pneumoniae	2	B	w	P
Methylobacterium mesophilicum	1	B	d	P
Ochrobactrum anthropii	2	G / B	w	P / R
Pantoea sp.	1 and 2	B	w	R
Pantoea spp.	1 and 2	B	d	M
Pseudomonas asplenii / putida	1	G	d / w	M / P
*Pseudomonas aeruginosa (*ATCC 101045*)*	2	G	w	P
Pseudomonas aeruginosa	2	B	w	P
Pseudomonas boreopolis	1	G	w	P
Pseudomonas fluorescens	1	B	w / d	P / R
Pseudomonas fluorescens / putida	1	B	w	R
Pseudomonas putida	1	B	w	R
Pseudomonas stutzeri	1	B	d / w	M / R
Pseudomonas sp.	1 und 2	G	d	M
Ralstonia sp.	1 and 2	G	w	P
Rhizobium sp./ *Agrobacterium* sp.	n. d., 1	G / B	d	M / P
Rhizobium sp. / *Agrobacterium* sp. / *Azospirillum* sp.	1	B	w	R
Roseomonas genomospecies	n. d.	B	w	P

Continuation of Table 2.2

Roseomonas sp.	n. d.	G	d	P
Candidatus Roseomonas massilieae	n. d., 1	G	d	M
Serratia marcescens	2	B	w	P
Sphingobacterium spiritivorum	2	B	w	P
Sphingobium cloacae	1	G	w	P
Sphingobium yanoikuyae	1	G	d	M / P
Sphingomonas paucimobilis	2	B	d / w	M /P
Sphingomonas sp. V1	1	G	d	M
Sphingopyxis chilensis	1	G	d	P
Stenotrophomonas maltophila	2	B	d / w	M / P / R
Stenotrophomonas maltophila, Stenotrophomonas sp.	2	G	d	P
Stenotrophomonas sp.	2	G	d	M
Vibrio metschnikovii	2	B	w	P
Gram positive strains				
Bacillus pumilus	E	G	d	P
Bacillus sp. CNJ905 PL04	E	G	w	R
Bacillus sp.	1 and 2	B	w	M
Bacillus sp. */ Lysinibacillus* sp.	n. d.	B	w	M
Bacillus thuringiensis	1, E	G	w	P
Cellulosimicrobium cellulans	n. d., E	G	w	R
Cellulosimicrobium cellulans / funkei	n. d.	B	w	M / P
Cellulosimicrobium sp.	1 and 2	B	w	R
Exiguobacterium sp. *India orange*	1, E	G	w	R
Exiguobacterium sp. BTAH1	1, E	G	w	R
Microbacterium aurum	1	G	d	M
Microbacterium liquefaciens, maritypum, oxydans	1	G	w	P
Microbacterium oxydans	1, E	G	w / d	P / M
Microbacterium paraoxydans	1	G	w	M
Microbacterium sp.	1 and 2	B / G	w / d	P / M
Microbacterium sp. SKJH-22	1	G	d	M
Mirococcus luteus	1	G	d / w	M / R
Paenibacillus sp. *(bacillus-relative)*	1 and 2	G	w	R
Rhodococcus fascians	1	G	d	M / P
Rhodococcus sp.	1 and 2	G	d	M / P
Rhodococcus sp.or *Nocardia* sp.	1 and 2	G	d	M / P
Staphylococcus cohnii subsp. *urealyticum*	1	G	d	M
Williamsia sp. KTR4	2	G	d	M
Yeast and filamentous fungi				
Alternaria (sterile mycelium)	n. d.	B	d	M
Alternaria	n. d.	B	w	P
Aspergillus ochraceus	n. d.	B	w	P
Aspergillus versicolor	n. d.	B	w	P
Capronia coronata	n. d.	G	d	M
Cladosporium sphaerospermum	n. d.	B	w	R
Cladosporium sp..	n. d.	B	d	P
Cryptococcus sp.	n. d.	G	d	M
Cryptococcus sp. HB949	1	G	d	M
Dematiaceaous (sterile mycelium)	n. d.	B	d	M

Continuation of Table 2.2

Dematiaceaous (sterile mycelium)	n. d.	B	d	M
Rhodotorula mucilaginosa	1; rare 2	G / B	d	P / R / M
Rhodotorula mucilaginosa AFTOL-ID 1548	1	G	w	P
Rhodotorula mucilaginosa AFTOL-ID	1	G	d	M
Rhodotorula mucilaginosa SJ 197	1	G	w	R
Rhodotorula slooffiae	1	G	d	M
Penicillium	n. d.	B	w	P
Penicillium (conidia)	n. d.	B	w	R
Penicillium sp.	1	B	w	P
Dermatiacea (phoma-like)	n. d.	B	d	M
Sphaeropsidales (phoma-like)	n. d.	B	d	M
sterile mycel, phoma-type	n. d.	B	d	M
Trichosporon domesticum	2	G	w	P

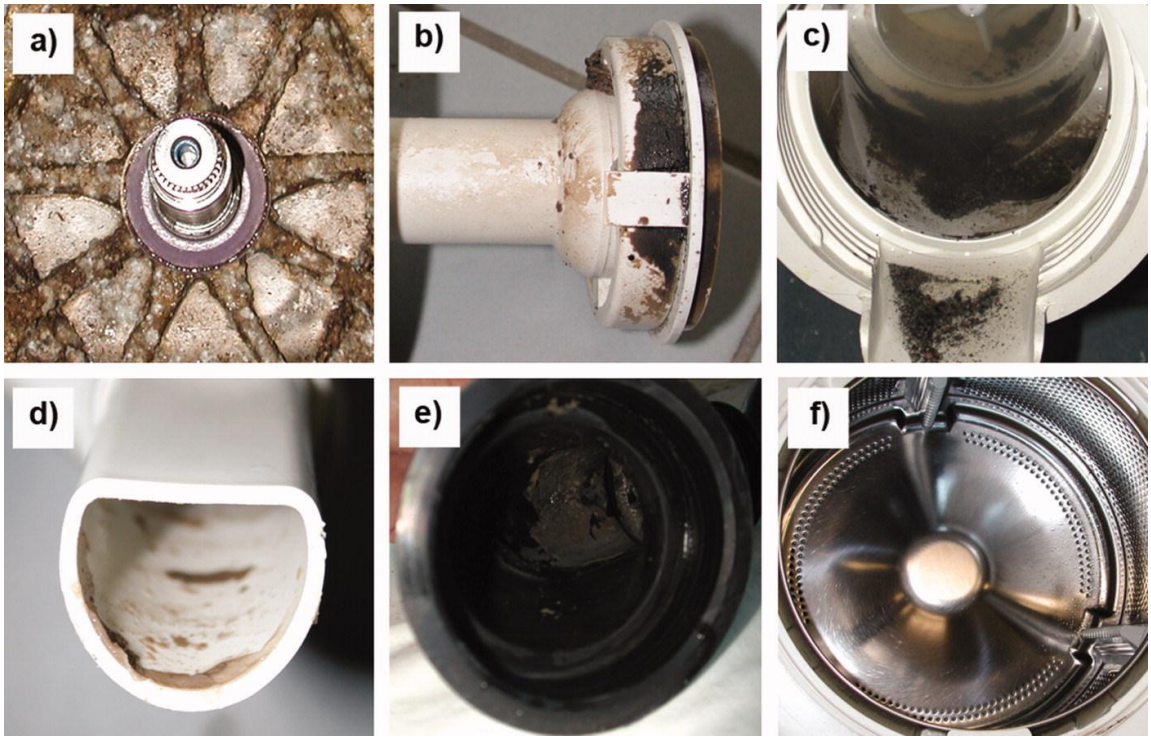

Figure 2.1. Parts of household washing machines prone to biofouling. Mainly hardly accessible, wetted parts of the washing machine were colonized. a = crossbar (metal alloy); b = filter lid (thermoplastics); c = filter (thermoplastics); d = air trapping (thermoplastics); e = rubber tube (rubber); f = inner drum (metal alloy) without biofilm formation.

2.1.3.2 The majority of the tested WM microorganisms formed more biofilm than the type strains

Fifteen strains were chosen that cover the three groups Gram-negative, Gram-positive bacteria and yeast, belonging to risk group 1 (exception *Citrobacter freundii)*. They were reactivated from the dried biofilm in the sampled washing machines and grown in complex and defined minimal media. In a series of experiments, the ability of WM isolates to form biofilm was quantified and compared to the one of their type strain. The initial cell loads were the same for the WM and the type strains, 10^5 and 10^6 cells mL^{-1} for yeast and bacteria, respectively. After growth for 24 h in biofilm minimal medium an increase in CV staining for nine of the WM isolates was observed in comparison to their reference counterparts (Figure 2.2). These results indicated that these nine isolates were forming more biofilm than their type strains. Out of the 15 WM isolates, nine formed more, two formed similar amounts, and four formed less biofilm than their reference counterpart obtained from the culture collection (Figure 2.2). The Gram-positive *Cellulosimicrobium* sp. WM (p-value: 1.7×10^{-42}) and *Microbacterium oxydans* WM (p-value: 2.3×10^{-42}), as well as the yeast *Rhodotorula mucilaginosa* (WM 1 and 3) (p-values: 3.2×10^{-26} and 9.4×10^{-6}, respectively) formed significantly more biofilm than their type strains. In contrast, *Rhodotorula minuta (Rhodotorula* sp. WM A, p-value: 1.7168×10^{-9}) and *Rhodotorula slooffiae* (*Rhodotorula* sp. WM B, p-value: 1.7×10^{-29}) formed significantly less biofilm than their type strain. The largest difference in biofilm formation was observed for *P. putida* and *Pseudomonas fluorescens*. *P. putida* WM from the washing machine produced twice as much biofilm as the reference strain from the collection. *P. putida* WM was also the best biofilm former of all the tested strains.

To support the findings of the CV assay, the EPS and cells of the *P. putida* biofilm were stained and observed with CLSM. These observations confirmed that *P. putida* WM formed greater amounts of EPS than the type strain (Figure 2.3). The cells of the WM isolates often appeared yellow (overlay of red and green), indicating that they were embedded in the EPS matrix. In the biofilm of the type strain the cells were mainly found at the bottom of the well and not protected by EPS.

2.1.3.3 Tolerance of 1-day-old *P. putida* biofilm towards washing detergents

CLSM observations of *P. putida* WM and the reference biofilms revealed that the highest concentration of IEC-A* detergent (7 g L^{-1}) removed the cells but not all the EPS

and cell debris (Figure 2.3). The remaining biological material was more abundant and dense for the WM isolate than for the type strain. The treatment with low concentrations of detergent only partially removed the cells and the EPS for both the type and WM strains. Tolerance towards detergent was evaluated in terms of biofilm removal by CV assay. After contact with 7 g L^{-1} of the IEC-A* detergent solution for 30 min, no remaining *P. putida* biofilms was detected by the CV assay. With lower concentrations of the detergent (0.0007 - 0.07 g L^{-1}) the CV signal was detected, indicating that the biofilms were not completely removed. The WM strain which formed more EPS was more tolerant than the type strain (Figure 2.4).

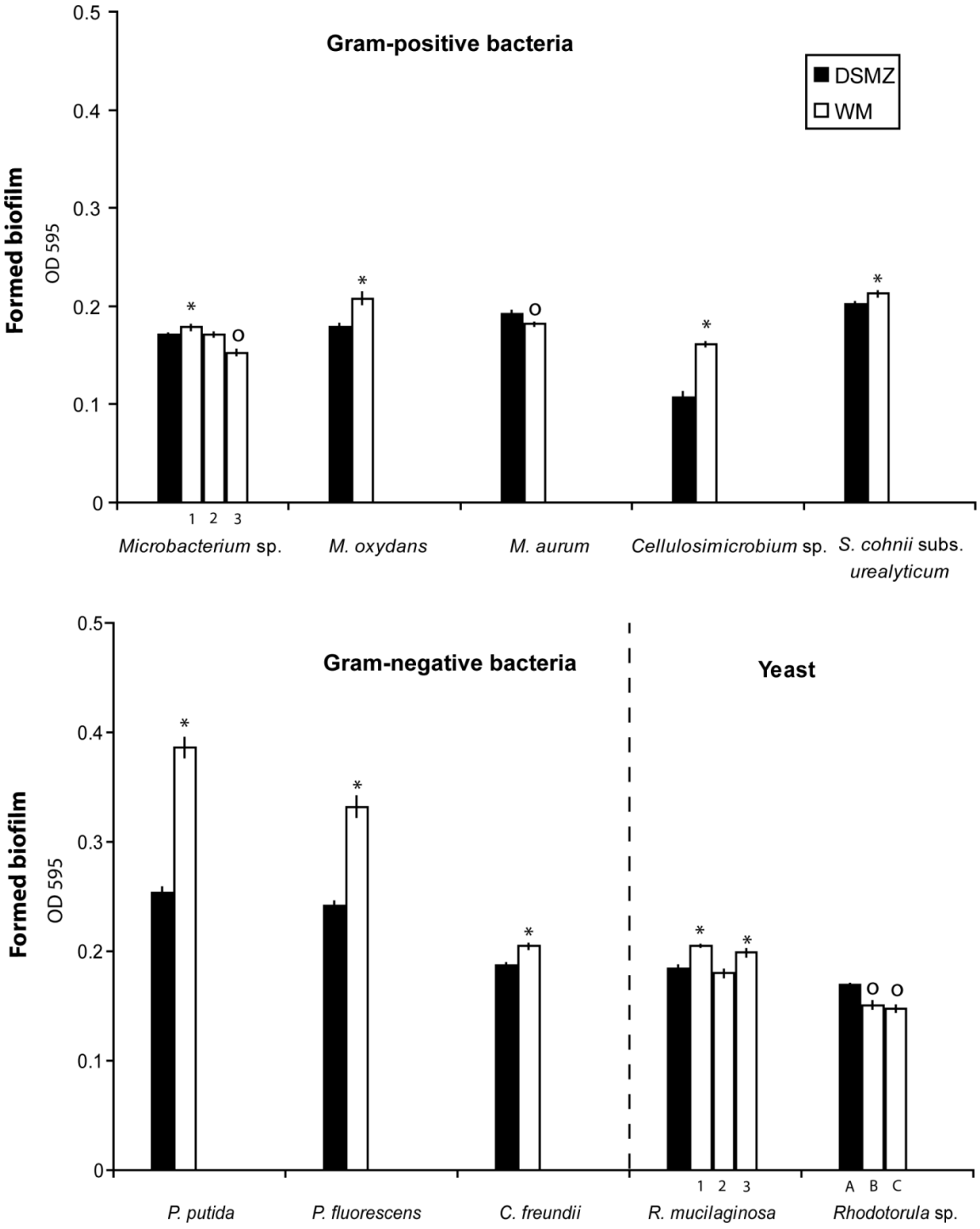

Figure 2.2. The biofilm formed by the washing machine isolates (□) and their type strains (▪) within 24 h (n = 378 wells, ± 95% confidence level). Formation of biofilm was measured by CV assay and is reported as the OD at 595 nm. (∗) and (o) indicate when WM isolates formed quantitatively more or less biofilm than type strains, respectively. A = *Rhodotorula minuta* (DSMZ 3016); B = *Rhodotorula minuta* WM; C = *Rhodotorula slooffiae* WM.

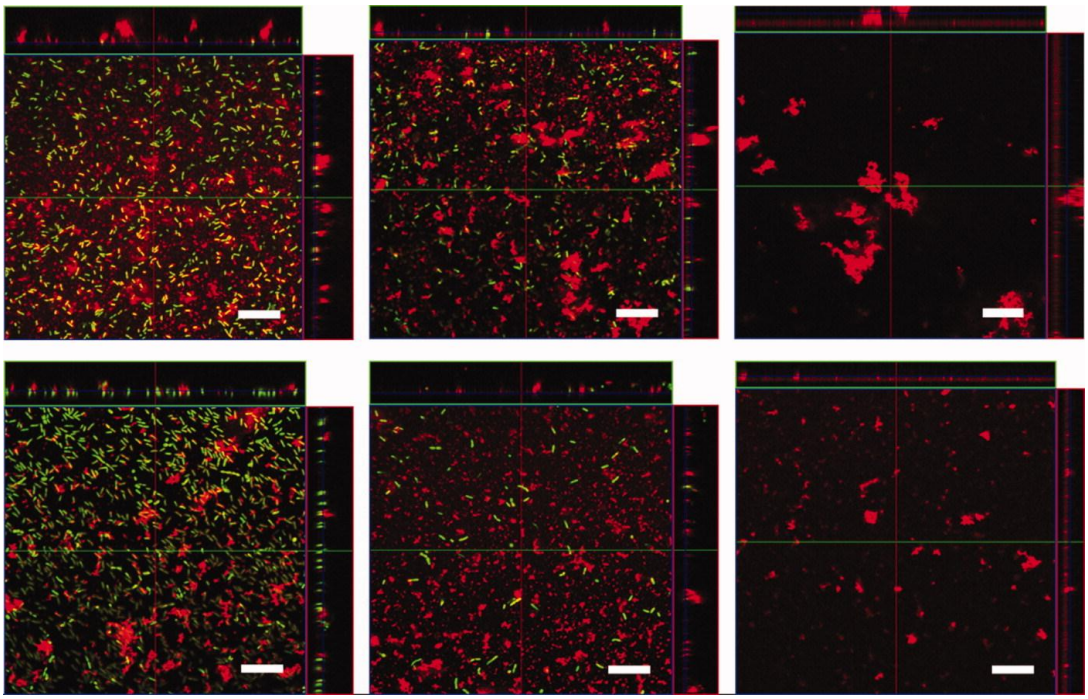

Figure 2.3. Overlay of confocal micrographs of 1-day-old biofilms of a *P. putida* strain isolated from a washing machine (upper panel) compared with its type strain (DSMZ 50026) (lower panel) after exposure to IEC-A* detergent at different concentrations: 0.0007 g L^{-1} (left), 0.07 g L^{-1} (middle) and 7 g L^{-1} (right). The DNA of the cells was stained with Syto BC (488 nm, green) and EPS was stained with Concanavalin-Alexa633 (632 nm, red). Scale bar = 20 μm.

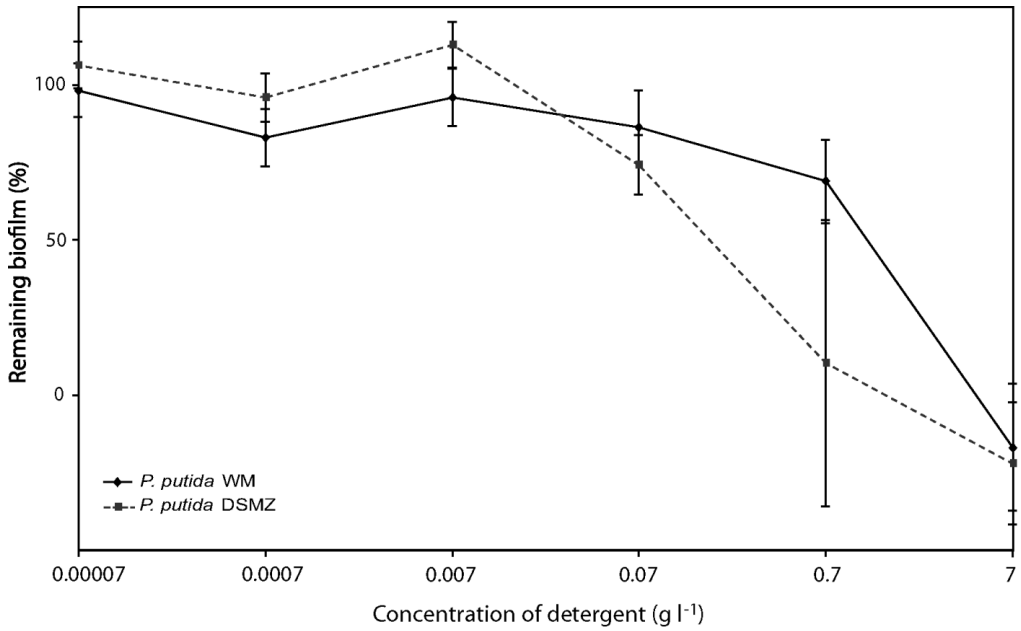

Figure 2.4. Effect of IEC-A* detergent concentration on *P. putida* biofilms. Reduction of biofilm measured as the average of OD value of CV staining and 95% confidence, n = 72 to 108. A *P. putida* strain isolated from a washing machine (WM) was compared with the type strain (DSMZ 50026).

2.1.4 Discussion

2.1.4.1 Formation of biofilm in washing machines

Ninety-four species isolated from washing machines from four countries were cultured and identified. The isolates were typical environmental microorganisms inhabiting soil, water and the human body, including, among others, members of the Enterobacteriaceae and Pseudomonadaceae. The majority of the WM isolates were also found on other domestic surfaces or in freshwater. On showerheads the predominant colonizers were *Mycobacterium* spp., *Staphylococcus* spp., *Escherichia* spp. and *Pseudomonas* spp. (Feazel et al. 2009), whereas *Methylobacterium* spp. and *Sphingomonas* spp. were the main colonizers of shower curtains (Kelley et al. 2004). These microorganisms were also found on kitchen sponges or dishrags (Enriquez et al. 1997; Michaels et al. 2003).

The identified microorganisms were mesophilic and fast growing on rich medium (TS or SD). About 30% of the microorganisms isolated from the washing machines belonged to potential human pathogens such as *P. aeruginosa* and *K. pneumoniae*. The percentage of potential pathogens (risk group 2) was found to be surprisingly high compared to the 3.6% of the 56 bacterial strains isolated from toilet bowls (Egert et al. 2010). In a healthy person, an infection with an opportunistic pathogen is controlled by the immune system. However, opportunistic pathogens are the main cause for morbidity and mortality in immunocompromised individuals (Brieland et al. 2000; Wanke et al. 2000).

Moreover, it was observed that the microbial composition varied depending on the geographical origin of the washing machine. Washing machines from South Korea comprised more fungi and yeasts than washing machines from Europe or the USA. The reason(s) for this observation could be (i) a different occurrence and distribution of microorganisms in the environment, (ii) different environmental conditions such as temperature or relative humidity and/or (iii) the use of different washing detergents and washing conditions.

The surfaces inhabited by microorganisms were not limited to permanently wet environments and comprised metal, rubber and plastics (typically polypropylene). Biofilms were never detected in the drum where the laundry is placed. However, rubber and plastic parts in direct contact with the operator were prone to biofilm formation. In general, biofilms developed to a larger extent in the inner parts of the washing machine and were hidden to the user's eyes. Considering these findings, it is recommended that precautions are taken, especially to limit the dispersion of the spores during dismantling of the washing

machine. Surprisingly, the detergent drawer, into which the highly concentrated washing detergent is added, was also prone to biofilm formation.

2.1.4.2 Tolerance of biofilm towards washing detergents

Detergents are primarily formulated to remove soil from clothes under dynamic conditions. In particular, bleach containing detergents are known to reduce the microbial load in washing machines (Terpstra 1998; Wilson et al. 2007). However, clothes are not the only place where microorganisms may be found and should be removed. Potential biofilm formation on mechanical parts of washing machine exposed to gentle mixing should also be considered. Although detergents were not designed for this particular purpose, the efficacy of a non-phosphate standard powder formulation containing bleach and bleach activator were tested against 1-day-old biofilm. The recommended concentration of the washing detergent IEC-A* (7 g L^{-1}) was insufficient to remove EPS and cell debris as demonstrated by microscopic observations. Even a 1-day-old biofilm could withstand relatively high concentrations of detergent. CV staining, which is less sensitive than direct microscopic observations, showed that half of the biofilm of a WM isolate could be removed at a detergent concentration between 0.7 g L^{-1} and 7 g L^{-1}. Concentrations that were 10 times lower were sufficient to remove the biofilm formed by the type strain. This indicated how inefficient detergents are against biofilms really growing inside washing machines. Numerous theories have been proposed to explain why biofilms are more tolerant to disinfection (Fux et al. 2005; Walker and Marsh 2007). Several studies even demonstrated resistance and/or adaptation towards sanitizers such as active chlorine compounds (Yildiz and Schoolnik 1999; Russell 2004) or quaternary ammonium bases (Sundheim et al. 1998; Langsrud et al. 2003). Experiments performed under real washing conditions have shown that the bleach component has the main impact on the survival of bacteria on textiles in both detergent solution and wastewater (Munk et al. 2001). Therefore, liquid detergents or powder formulations lacking bleach will most likely have a lower efficacy of biofilm removal. Beadle and Verran (1999) have already shown that liquid detergents without bleach allow the recovery and growth of microorganisms in a low nutrient environment.

P. putida WM produced more biofilm and appeared to be more tolerant towards detergent than its type strain. This observation is in agreement with other studies reporting that a larger amount of biofilm leads to better protection of the cells (Davies et al. 1998; Cochran

et al. 2000). Microscopy showed that 7 g L^{-1} of IEC-A* detergent was sufficient to remove the cells but a lot of EPS was still present. However, the amount of remaining biofilm was not sufficient to be detected by CV staining. Antoniou and Frank (2005) showed that biofilms of *P. putida* treated with different concentrations of NaOH at 66°C for 3 min reduced the cellular coverage on surfaces more easily than the polysaccharide/EPS coverage. Deposition of organic materials such as EPS is problematic because it serves as site for attachment of other organisms including cells which are not able to produce EPS (Neu 1992; Gomez-Suarez et al. 2002). Among all the constituent of EPS, the carbohydrates are the most relevant in term of bacterial attachment (Jain and Bholse 2009). Since the EPS may help the cells to re-colonize the surface the detergent should not only remove the cells but also the EPS.

2.1.5 Conclusions

Microorganisms were able to form biofilms on diverse materials and locations within household washing machines. Tests under laboratory conditions demonstrated that the recommended concentration of a standard powder formulation (IEC-A*) was not sufficient to entirely remove a 1-day-old biofilm. These findings question the validity of the standard procedures based on planktonic cells to test the efficacy of washing detergents on biofilm removal. To be more representative, it is recommended, firstly that tests with biofilms are to be conducted because this is the main form of bacterial life in a household washing machine, and secondly that microorganisms isolated from washing machines are to be used because they are already adapted to chemical and mechanical stresses. Better knowledge of tolerance and adaptation to washing detergents will also help to improve the efficiency of detergents as well as washing programs towards biofilm removal. This is especially of interest because washing behaviour has changed a lot in the last few years. In particular, the user tends to wash at low-temperature and with bleach-free detergents. The influence of these changes on formation of biofilms in the washing machine is still unknown.

Acknowledgements

This work was granted by the Swiss agency for innovation (CTI project 9075.1PFLS-LS). The authors thank V-Zug, Samsung and Miele for their technical and financial contribution. Special thanks go to Monika Braegger and Bettina Lanz for their support during strain isolation and identification, Aline Hunziker for help during biofilm screening, Isabel Hartmann for her advice on the crystal violet assay, Dr. Jean-Pierre Kaiser for support regarding the CLSM, Dr. Julian Ihssen, Bruno Wampfler, Prof. Dr. Leo Eberl and PD Dr. Dr. h.c. Linda Thöny-Meyer for fruitful discussions.

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2.2 Supplementary data - Chapter 2 (not published)

The following sections comprise unpublished experiments which are thematically linked to the previous paragraph (2.1 Biofilms isolated from washing machines from three continents and their tolerance to a standard detergent).

These experiments were performed to cover several aspects of the behavior of microbes isolated from washing machines.

Shake flask experiments with selected washing machine isolates were performed to analyze the planktonic cell growth in different cultivation media as well as biofilm formation on differently preconditioned polystyrene surfaces.

To determine the removal efficiency of a standard detergent on the yeast *Rhodotorula mucilaginosa* and the Gram-positive strain *Staphylococcus cohnii* subsp. *urealyticum* washing tests were performed in analogy to the Gram-negative strain *P. putida.*

The viability of *P. putida* cells after treatment with the standard detergent IEC-A* was analyzed using a viability stain (INT) to determine if cell viability is affected by the the detergent. This experiment is completing the washing test performed with the CV assay in paragraph 2.1.

Further, each of the three components of the standard washing detergent IEC-A* was tested seperately to determine its ability to remove biofilms. For this purpose a strong biofilm former, *E. coli* PHL628, and the washing machine isolate *Pseudomonas fluorescens* were used.

My contribution was the performance of the growth experiments in shake flasks with the different strains, the washing tests with *Rhodotorula mucilaginosa* and *Staphylococcus cohnii* subsp. *urealyticum* and the attachment test and the test with the decomposition of the standard detergent. Dr. L. Mauclaire conducted the INT assay with *Pseudomonas putida* WM and DSMZ. Plasma treatments for the attachment test were conducted by Sébastien Guimond. The IEC-A base detergents used in the described experiments is also referred as IEC-A* base detergent (WFK Testgewebe GmbH).

2.2.1 Planktonic cell growth of washing machine isolates

2.2.1.1 Introduction

The aim of this experiment was to screen the washing machine isolates to find the most appropriate species as model biofilms. For this purpose, ninety-four isolates have been selected from the most frequent bacteria and yeasts found in eleven analysed washing machines from three continents (Chapter 2, Table 2.2). From the 94 isolates, 22 strains covering the Gram-positive, Gram-negative bacteria and yeasts were chosen of which 17 strains were used for planktonic growth experiments in shake flasks to study their behaviour in a complete medium and three minimal culture media using glycerol as carbon source.

2.2.1.2 Materials and Methods

Cell cultivation

The selected microorganisms were grown on 4 different culture media: i) nutrient broth (5 g L^{-1} peptone, 3 g L^{-1} meat extract, pH 7), which was the most appropriate medium for most of the strains (DSMZ), ii) E minimal medium (3.5 g L^{-1} $NaNH_4HPO_4$ x 4 H_2O, 3.7 g L^{-1} KH_2PO_4, 7.5 g L^{-1} K_2HPO_4, 4 g L^{-1} glycerol and with 1 mL of sterile-filtered $MgSO_4$ (234.5 g L^{-1} $MgSO_4$ x $7H_20$) and 1 mL sterile-filtered trace element stock solution (2.78 g L^{-1} $FeSO_4$ x 7 H_2O, 1.47 g L^{-1} $CaCl_2$ x $2H_2O$, 1.98 g L^{-1} $MnCl_2$ x 4 H_2O, 2.38 g L^{-1} $CoCl_2$ x $6H_2O$, 0.17 g L^{-1} $CuCl_2$ x 2 H_2O, 0.29 g L^{-1} $ZnSO_4$ x $7H_2O$ in 1 L 1 M HCl added after autoclaving, pH = 7.1), iii) E minimal medium with 3 g L^{-1} tryptic soy broth (TSB) replacing the glycerol and iv) biofilm minimal medium (1 g L^{-1} MOPS, 1.1 g L^{-1} $(NH_4)_2SO_4$, 0.15 g L^{-1} KH_2PO_4, 0.25 g L^{-1} $MgSO_4$ x 7 H_2O, 0.1 g L^{-1} $FeSO_4$ x $7H_2O$, 0.2 g L^{-1} EDTA, 4 g L^{-1} glycerol and 1 mL of sterile filtered trace element stock solution (1.5 g L^{-1} $CaCl_2$ x $2H_2O$, 3.96 g L^{-1} $MnCl_2$ x $4H_2O$, 5.62 g L^{-1} $CoSO_4$ x $7H_2O$, 0.34 g L^{-1} $CuCl_2$ x $2H_2O$, 1 g L^{-1}, $ZnSO_4$ x $7H_2O$ 1 g L^{-1}, MoO_4Na_2 x $2H_2O$, pH = 1).

Cryo cultures were grown overnight in 8 mL of 50% NB, 50% tested medium and transferred (dilution 1:100) in 300 mL of corresponding medium in Erlenmeyer flasks, incubated at 30°C and shaken at 150 rpm. Growth was followed by the measurement of optical density at 600 nm.

2.2.1.3 Results and Discussion

Planktonic cell growth of washing machine isolates

In the literature, the yeast strain *Saccharomyces cerevisiae* is well known to attach to other cells (to form flocs) and also to surfaces (Reynolds et al. 2001). The tested yeast strains (*Rhodotorula* spp.) had the ability to form biofilms on glass. However, in the shake flask experiments, most of the yeast cells started to flocculate when the medium was supplemented with glycerol (Table S2.1). Bayly and co-authors (Bayly et al. 2005) reported that nutritional components influences the flocculation ability. Also bacterial strains like *Burkholderia cepacia* are able to form flocs but in contrast to the yeast cells, flocs could be observed in all tested culture media. The cell number in the culture could not be measured accurately although the flocs were resuspended and sonified. In case of yeast cells, the formation of flocs is a stress response mechanism to adverse conditions (Claro et al. 2007).

Williamsia muralis did not grow well in any of the four different culture media. *W. muralis* also grew very slow on nutrient agar plates. It was observed that several of the 90 isolates from washing machines could not be recultivated, probably due to impaired fitness.

The biofilm minimal medium was designed to trigger biofilm formation during the main bioprocess in biofilm reactors. However, it was not the appropriate medium to promote cell division of planktonic cells. Considering biofilm production of the model biofilm in biofilm reactors, complete medium (growth medium 1) will be used for the proliferation of planktonic cells before triggering biofilm formation with a low nutrient medium.

Table S2.1. Planktonic growth of the 17 washing machine isolates. Signs indicate the extent of growth based on optical density measurements at 600 nm: (-) < 0.2; 0.2< (0) < 0.5; 0.5< (+) < 1; 1 < (++) < 1.5; 1.5 < (+++) < 2, (++++)>2. n. d.: not determined

Microorganism Strain	Growth medium 1	Growth medium 2	Growth medium 3	Growth medium 4	Biofilm	Flocculation
Gram-negative						
Pseudomonas putida T1/2	++++	++++	+	0	Yes	Yes
Pseudomonas fluorescens T1/1	++++	+++	+	+	n. d.	n. d.
Citrobacter freundii	++++	+++	+	+	Yes	n. d.
Burkholderia cepacia	n. d.	-		-	Yes	Yes
Gram-positive						
Microbacterium oxydans G2/1	++	++	-	-	Yes	n. d.
*Microbacterium oxydans.*G1/1b	++	-	++	-	Yes	n. d.
Microbacterium sp. T2/11	++	-	+	0	Yes	n. d.
Microbacterium sp. G1/5	-	-	-	-	n.d	n. d.
Cellulosimicrobium sp.	++	+++	++	+	n.d	n. d.
Rhodococcus sp. G1/17	+++	-	++	-	n.d	n. d.
Rhodococcus sp. G2/17	+++	-	++	0	n.d	n. d.
Williamsia muralis	-	-	-	-	n.d	n. d.
Yeast						
Cryptococcus sp.	++	-	+	-	Yes	Yes
Rhodotorula mucilaginosa K10 1548	++	-	+	-	Yes	Yes
Rhodotorula mucilaginosa K10	++	n. d.	++	-	n. d.	Yes
Rhodotorula mucilaginosa K9	++	0	+	0	Yes	n. d.
Rhodotorula slooffiae K2	++	-	0	-	Yes	Yes

2.2.1.4 Conclusion

With this simple growth study conducted in shake flasks, it was possible to determine the growth behavior of selected washing machine isolates. *B. cepacia*, *Cryptococcus* sp. and *W. muralis* were not considered as adequate candidates for further tests. Either the extensively formed flocs could not be dissolved properly (*Cryptococcus* sp. and *B. cepacia*) or showed poor growth performance (*W. muralis*).

High nutrient conditions promote cell division very well and can be applied for batch cultivation in biofilm reactors before the main biofilm production process in low nutrient environment will be conducted to increase the probability of attaching cells to a surface.

Reference

Bayly JC, Douglas LM, Pretorius IS, Bauer FF, Dranginis AM. 2005. Characteristics of Flo11-dependent flocculation in *Saccharomyces cerevisiae*. FEMS Yeast Res 5:1151–1156.

Claro FB, Rijsbrack K, Soares EV. 2007. Flocculation onset in *Saccharomyces cerevisiae*: effect of ethanol, heat and osmotic stress. J Appl Microbiol 102:693-700.

2.2.2 Attachment test

2.2.2.1 Introduction

Attachment of cells onto surfaces is the most critical step in initializing biofilm formation (Palmer et al. 2007). Bacteria and yeast have the ability to form biofilms on different surfaces. According to the literature, they prefer rough surfaces (Carlen et al. 2001) and are able to grow on hydrophilic (Chavant et al. 2002) and/or hydrophobic surfaces (Cerca et al. 2005). However, not all pristine surfaces are appropriate for cell attachment, e.g. electrostatic repulsion between the cell and the surface can complicate direct attachment to the material. Therefore, conditioning films generated with organic and anorganic molecules from the surrouniding bulk fluid have the ability to change surface properties. Pre-conditioning of surfaces e.g. with proteins can: i) promote the attachment of cells onto a surface, functioning as linker between the cell and the surface or ii) have the opposite effect, acting as a competitor for surface binding sites.

The goal of the following experiment was to test five preconditioning strategies (bovine serum albumin, gelatine, mussle juice, and plasma treatment with Ar/O_2 or N_2 plasma treatment for the surface of polystyrene microtiter plates (TPP) and assess their ability to enhance cell attachment and following biofilm formation.

2.2.2.2 Materials and Methods

Preconditioning of microtiter plates

For the preconditioning, five different agents/methods were chosen: mussel juice (Herrera et al. 2007), bovine serum albumin (BSA; Fletscher 1976), gelatine (Fletcher 1976) and two types of plasma treatments either with Ar/O_2- and N_2-gas (Wan et al. 2003).

Mussel juice. Frozen mussels were cooked for 20 min. The supernatant (24.5 mL) was collected aseptically in a sterile falcon tube. About 28 µL were transferred into each well (n = 4 - 5 wells per well plate served as negative control and reference, respectively and were exposed to culture medium without microbial cells).

Bovine serum albumin (BSA). Ten mg BSA/100 mL H_2O (MilliQ) were prepared. Forty µl were pipetted in every well. The microtiter plates with the protein were dried in the sterile bench. After drying, the wells were filled with 200 µl of sterile-filtered distilled

water. The water remained in the wells for 1h to wash out the unbound proteins (n = 11 wells served as reference). The plates were dried again in the sterile bench.

Gelatine. 0.1% (w v^{-1}) gelatine in H_2O (MilliQ) was prepared and autoclaved. Forty µl were transferred into each well. The wells were dried in the laminar flow bench under aseptic conditions (n = 11 wells served as reference).

Plasma treatments. A set of well plates was plasma treated with Ar/O_2 resulting in a surface with O-based functional groups (-OH, -COOH etc). The other set was treated with N_2-based plasma with N-based functional groups (e.g. NH_3, amine, amide).

The plasma treated well plates are stable for at least 2 days when they are covered and are automatically sterilized by the plasma treatment. To assure sterility, the well plates were sterilised by UV light prior to usage for ca. 10 min in the sterile bench.

Polystyrene (negative control). Microorganisms were directly incubated in untreated well plates.

The experiments with the well plates with different treatments were performed in triplicates (42 wells per organism). For the negative control only one plate was used (n = 14 wells per organism).

Test organisms

The organisms for testing the influence of the surface modifications were a) isolated from household washing machines (*Pseudomonas putida*, *Pseudomonas fluorescens*, *Citrobacter freundii*, *Microbacterium oxydans*, *Microbacterium* sp. (3 strains), *Rhodotorula mucilaginosa* (3 strains) *Rhodotorula slooffiae*, *Rhodococcus* sp.) or b) being from the American type culture collection (*Candida albicans*), or from the own strain collection like *E. coli* PHL628 (Brombacher et al. 2006), *Micrococcus luteus* (provided by Paolo Landini). Cryo cultures of slow growing organisms were inoculated two days before (e.g. *Rhodotorula* sp., *Rhodococcus* sp., *Microbacterium* sp.). For faster growing organisms overnight cultivation was sufficient. All cultures were incubated at 30°C, 150 rpm. Yeasts were grown in SDB and bacteria in TSB.

Cultivation

The inoculum of each strain was diluted in biofilm minimal medium (Chapter 2) to an initial OD_{600} of 0.04 for Gram-negative, 0.05 for Gram-positive bacteria and 0.025 for

yeast. The cell number per mL was ~10^6 to 10^7 per mL for yeast and bacteria, respectively. Two hundred µl were transferred into twelve wells. Eleven wells were filled only with culture medium and served as blank. The experiment was performed in triplicates. The cells were incubated at 30°C and 100 rpm for 22.5 h.

Biofilm quantification

After growth, the 100 µl of the supernatant was transferred into fresh well plates (Nunc). The OD was measured at 595 nm with a plate reader (ELx800). For the biofilm quantification the crystal violet (CV) method was performed (as previously described in Chapter 2). The CV dissolved in 100% DMSO and measured at 595 nm.

Data analysis

The average value of the blanks (of all plates with the same treatment) was subtracted from each measurement and the average and standard deviation was determined. This was done for the OD measurement of the suspension and for the biofilm. The ratio between biofilm formation and cell suspension was determined as OD_{595} from CV assay divided by OD_{595} of the supernatant.

2.2.2.3 Results and Discussion

The biofilms with the differently treated polystyrene surface of microtiter plates were analyzed after 24 hours to determine the influence of conditioning films on cell attachment and the succeeding biofilm formation.

For *P. putida,* the negative control gave the highest amount of biofilms. The pre-conditioning of the surface did not enhance cell attachment. The effect of the pre-conditioning films on *P. putida* biofilms was either not detectable (gelatine, mussle juice and N_2 plasma) or was negative (BSA and Ar/O_2 plasma) (Figure S2.1, top).

Compared to all the other treatments, the Gram-positive strain *Microbacterium* sp. (Figure S2.1, middle) showed increased biofilm formation only in presence of mussel juice. Plasma-treated wells containing O-functional groups reduced the amount of

Microbacaterium sp. biofilm while BSA, gelatine and N_2-gas showed no significant enhancement of biofilm formation compared to the negative control.
In case of the yeast strain *Rhodotorula mucilaginosa* (Figure S2.1, bottom) none of the tested surface modifications increased the amount of biofilm in comparison to the uncoated control. Plasma treatment with the N_2-gas diminished biofilm formation.
The unmodified polystyrene surface exposed to the minimal medium was adequate for biofilm formation. The conditioning film that was built by the compounds in the medium was sufficiently efficient for cell attachment and did not require additional surface modifications.

For *E. coli* PHL628, which produced dense biofilms, the different preconditioning films increased biofilm formation, while for most of the bacterial strains only small differences could be observed (data not shown).

In literature, it has already been reported that preconditioning could have a negative influence on cell attachment, e.g. proteins building conditioning films could hinder the direct attachment of the cells acting as competitors for binding sites (Palmer et al. 2007) or additionally operate as antiadhesives preventing biofilm formation (Munk et al. 2008). On the other hand, it was stated that proteinasceous conditioning films could support biofilm formation, because the proteins serve as nutrients (Jeong and Frank 1994). This explanation applies only to the nutrient supply. However, biofilm formation showed no siginificant increase.
Interestingly, plasma treatment with addition of O-functional groups reduced biofilm formation for all three microorganisms, whereas wells containing N-functional groups did not enhance biofilm formation. The ability to promote cell adhesion of N-functional groups was only been described for animal cells (Dekker et al. 1991). The enhancement of cell adherence might not be applicable for microbial cells.
The optical density of the supernatant was also measured to determine the influence of the preconditioning film on the relative amount of planktonic cells. The biofilm/suspension ratio indicated, whether biofilm formation or planktonic cell growth predominated after a tested preconditioning treatment.
In several cases, the treatment did not show an increase in biofilm formation. The maximal biofilm/suspension ratio was found in the untreated well plates. We assumed that in this

case, the additional proteins rather served as nutrients and promoted planktonic cell growth than biofilm formation.

In summary, the attachment assay demonstrated the non-importance of the analyzed surface treatments for biofilm formation after 24 hours. Hence, for future experiments, no additional chemical surface treatment will be considered due to the low effect on biofilm formation.

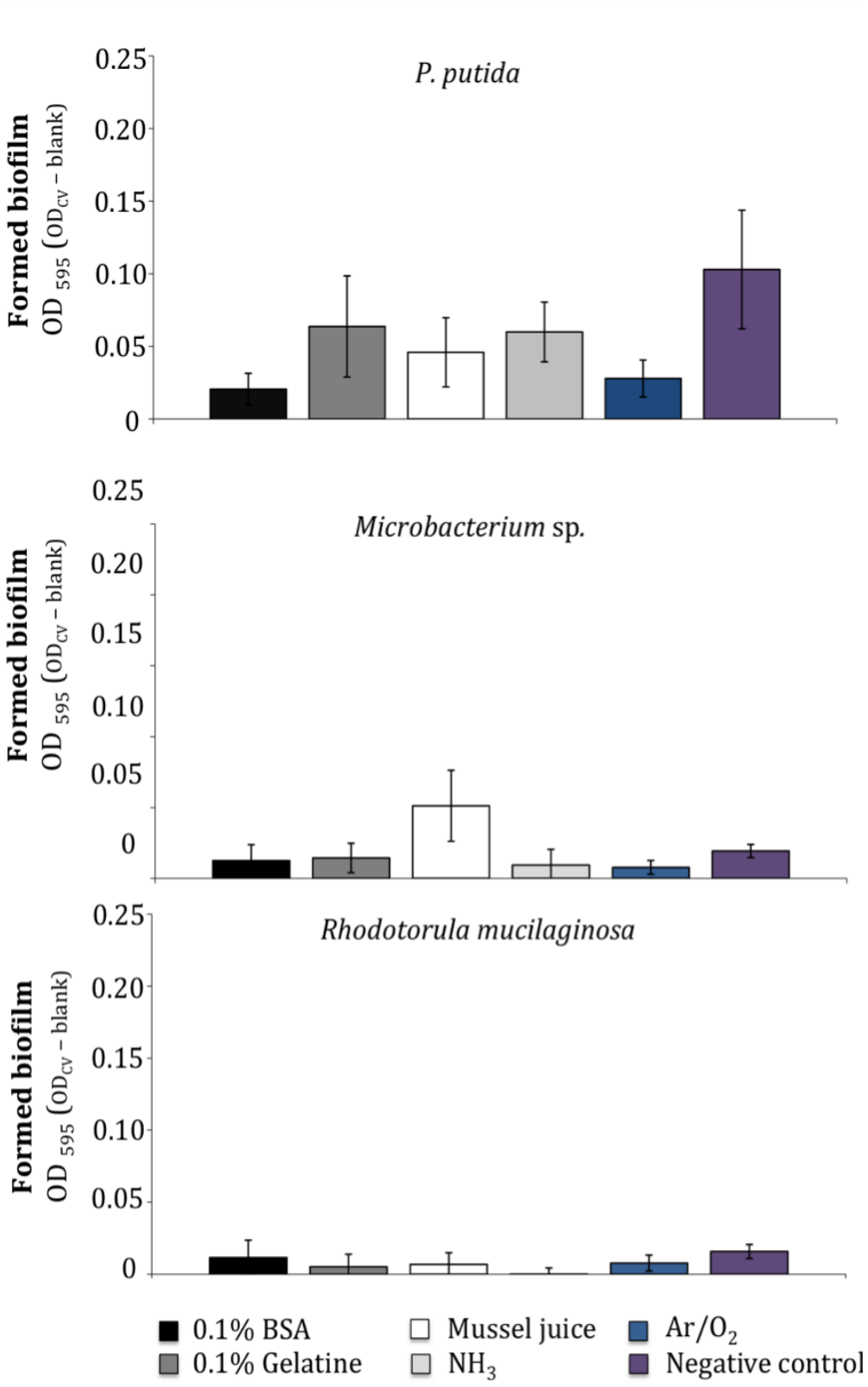

Figure S2.1. A representative of a Gram-negative (*P. putida*), Gram-positive (*Microbacterium* sp.) and a yeast (*Rhodotorula mucilaginosa*) strain growing on microtiter plates with all tested pre-conditioning methods. The formation of biofilm was measured by CV assay and is reported as OD at 595 nm with substracted blank (n = 42 wells).

2.2.2.4 Conclusion

This assay illustrated the diversity of cell interactions with various surfaces. However, pre-conditioning of the polystyrene well plates showed, that the different protein-based films as well as plasma-functionalization did not enhance the amount of attached cells and biofilms after 24 h.

The different surface modifications resulted in poorer or equal amount of biofilms after 24 hours in comparison to the untreated polystyrene. The effect of a pre-conditioning is strain-dependent and cannot be assumed for another one (e.g. mussel juice had a positive impact only on biofilms of *Microbacterium* sp.). Because cell surface characteristics among microorganisms are variable, an adjustment of surface conditioning for each single strain will be needed.

Reference

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2.2.3 Washing tests with *Rhodotorula mucilaginosa* and *Staphylococcus cohnii*

2.2.3.1 Introduction

Rhodotorula mucilaginosa and *Staphylococcus cohnii* subsp. *urealyticum* are representatives of the yeasts and Gram-positive bacteria growing in household washing machines. Besides *P. putida,* they belonged to the strains which were better biofilm former in comparison to their reference strain (Chapter 2.1). The effect of the standard detergent IEC-A* upon biofilm removal was already achieved for *Pseudomonas putida* and its reference strain. The washing machine isolate of *P. putida* was more tolerant towards biofilm removal as more remaining biomass was detected with the CV assay in comparison to its reference. The goal of the washing tests with *R. mucilaginosa* and *S. cohnii* was to examine if the standard detergent IEC-A* provoked similar effects as observed with *P. putida*.

2.2.3.2 Materials and Method

The strains

The cultivation and preparation of *R. mucilaginosa* and *S. cohnii* subsp. *urealitycum* was conducted as described in 2.1.2.3. The initial cell number of the yeast strain was adjusted to 10^5 cells per mL while for the Gram-positive strain it was set to 10^6 cells per mL by flow cytometry.

Washing detergent assay against biofilm in 96-well plates

The detergent test and the statistical analysis was conducted as described in 2.1.2.4. Biofilm removal was analyzed with CV assay as described in 2.1.2.3.

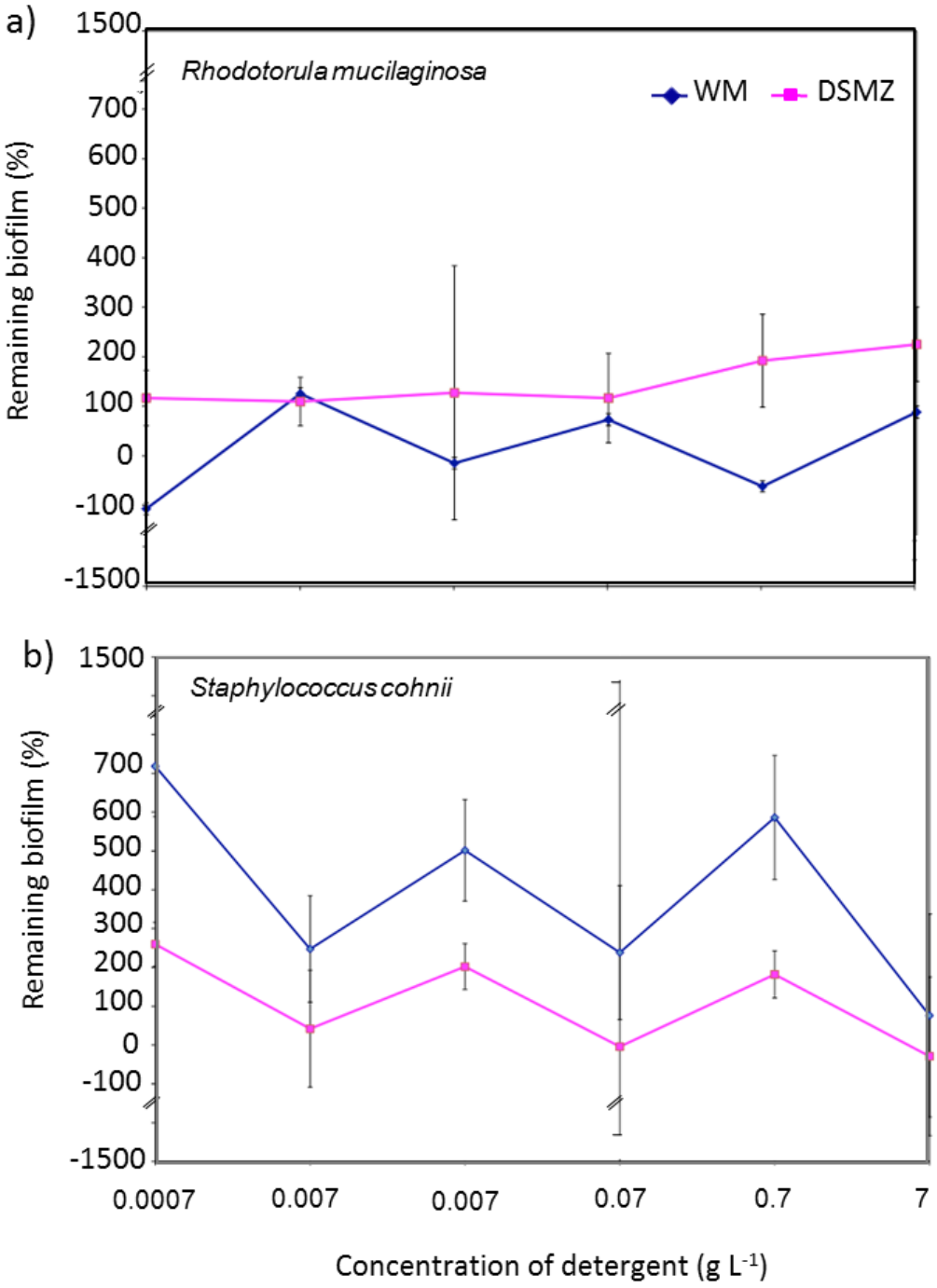

Figure S2.2. The biofilm removal efficiency of the standard detergent IEC-A* towards the a) yeast *R. mucilaginosa* and b) the Gram-positive strain *S. cohnii* subsp. *urealitycum* was tested in 96-well plates.

2.2.3.3 Results and Discussion

For the *S. cohnii* subsp. *urealyticum* and *R. mucilaginosa* (Figure S2.2), the results are not as clear as for *P. putida* (see Figure 2.4). In direct comparison, the reference strain of *Staphylococcus cohnii* subsp. *urealyticum* showed better tolerance towards detergents than the washing machine isolate. In case of *Rhodotorula mucilaginosa*, the washing machine isolate was more tolerant than its reference strain.

However, in comparison to *P. putida* less biofilm was produced by *R. mucilaginosa* and *S. cohnii.* Therefore, the difference between "before" and "after" the detergent treatment led to relatively high variations. A concentration-dependent biofilm removal with the washing detergent could not be observed for *R. mucilaginosa* and *S. cohnii* in comparison to *P. putida* and its reference strain. It was assumed, that higher amount of initial biofilms (before testing different detergent concentrations) would have led to a better performance of the biofilm removal test.

2.2.3.4 Conclusion

Regarding detergent efficiency test with the washing machine isolates *R. mucilaginosa* and *S. cohnii*, no similar washing behavior was observered as with *P. putida*. It was assumed that low initial biofilm concentration resulted in inconsistent biofilm removal.

Thus, to perform biofilm removal assays in 96-well plates, a minimal amount of biofilm needs to be present to receive reliable results. Thus, a slight modification of test set-up, either by chosing a better substrate material or adjusting the culture medium to increase biofilm formation for each test strain, can improve the performance of the removal test.

2.2.4 INT versus CV to determine the efficiency of biofilm removal

2.2.4.1 Introduction

The CV assay is a widely used method to obtain the relative biomass of miroorganisms. The CV assay is often applyied to determine the ability of certain active substances (e.g. detergents or antibiotics) to remove biofilms, staining the remaining biofilm. CV usually provides information about the relative biomass, i.e. including cells and EPS matrix but not about the viability of cells.

The INT assay was performed to assess the respiration activity of cells after treatment with the standard detergent IEC-A*. The INT assay complements the experiment in paragraph 2.1 applying the CV assay to define biofilm removal ability of the standard detergent IEC-A*.

2.2.4.2 Materials and Method

Cell activity in presence of washing detergents

Washing detergent was tested on 24 h-old biofilm of *P. putida* WM and *P. putida* DSMZ and produced as described in 2.12.4. Washing detergent (5.39 g L^{-1} IEC-A base, 1.4 g L^{-1} Na-perborate, 0.21 g L^{-1} tetra acetylethlyene diamine (TAED)) was dissolved in cold tap water and constituted the 100% fresh stock solution. To obtain different concentrations, the stock solution was diluted with cold tap water in 10-fold dilution steps (10 - 0.01%). Two hundred µL of washing detergent was added in each well for simulated washing process (30°C, 50 rpm for 30 min) (see 2.2.4). The wells were rinsed five times, dried in the sterile bench. Removal of biofilm was evaluated by CV staining (0.1%) according to the protocol already described in this chapter (2.2.3).

The cell respiration activity within the *P. putida* biofilm was estimated using INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride). Dried microtiter plates were loaded with 0.2 mL of biofilm minimal medium containing 1 g L^{-1} glucose and 0.5 g L^{-1} INT (previously dissolved in methanol and added to 0.1 M Tris buffer, pH = 7). Plates were placed in the dark at 30°C and 50 rpm. After 4 hours the supernatant was discarded and formazan salts were dissolved in 0.2 mL DMSO (60 hours). One hundred µL were transferred into fresh 96-well plates (Nunc) and absorbance was measured at 460 nm.

Experiments were conducted in triplicates. The respiration activity was calculated using 0.0001% detergent concentration as reference value.

2.2.4.3 Results and Discussion

According to the CV assay, *P. putida* (WM) produced more biofilm than the reference strain under similar starting conditions and it also appeared to be more tolerant towards the washing detergent. Other studies have already reported that a higher amount of cells leads to better preservation of the biofilm (Davies et al. 1998; Cochran et al. 2000).

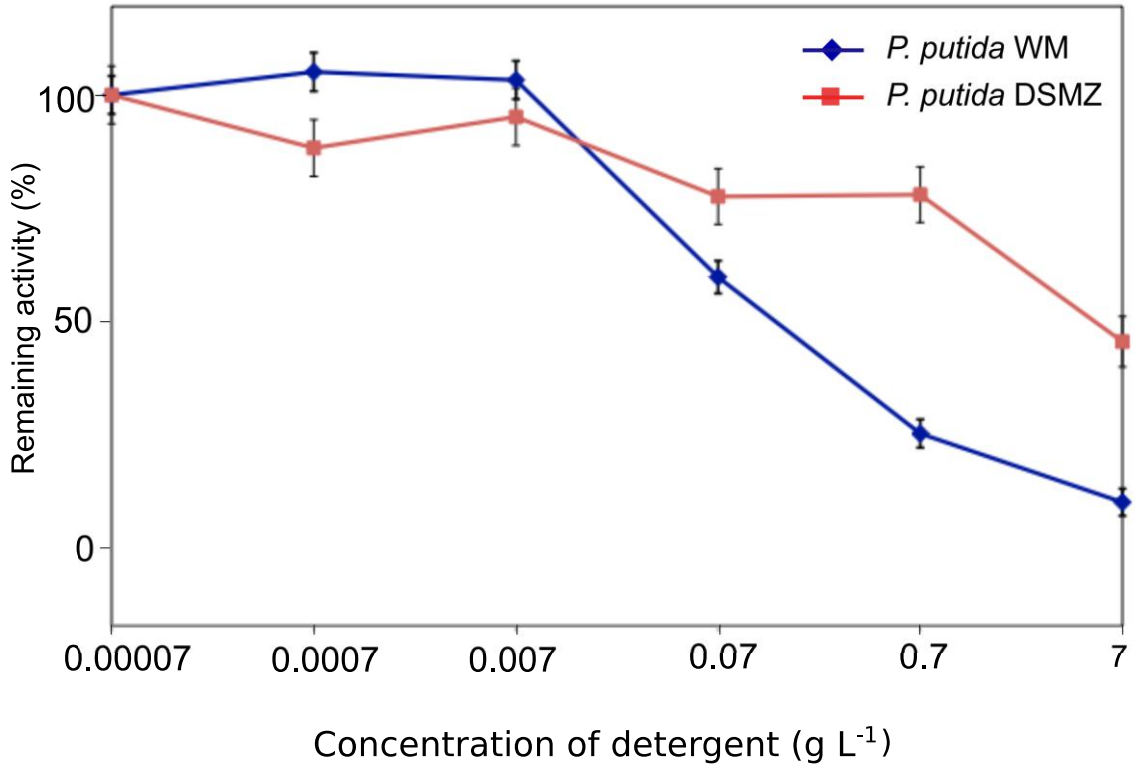

Figure S2.3. Respiration activity (measured by INT) after simulated washing of biofilm with 0.001 to 100% concentration of detergent. A *P. putida* strain isolated from a washing machine was compared with the reference strain (DSMZ50026). Average values of triplicate experiments conducted with 3 clones ± 95% confidence level (n = 3 x 36 wells).

However, regarding respiratory activity, the reference strain was more active than the WM isolate (Figure S2.1). Two hypotheses could be formulated to explain this finding. First, we observed that the reference strain had a lower respiratory activity than the WM strain. In general, cells with low activity have the better ability to deal with stresses such as

antimicrobial agents (Brown et al. 1988). The second hypothesis is related to the 3D structure of the biofilm as revealed by CLSM observations. With microscopy we observed that the cells in the WM biofilm were mainly located in close vicinity to the EPS. After washing, clumps of biofilm were detaching and floating in the suspension. Therefore, the majority of the active cells were removed by washing. By contrast, the cells of the reference strain were found mainly at the bottom where they could be better protected against the washing. Huang and colleagues (Huang et al. 1995) demonstrated the non-uniform spatial loss of respiratory activity of biofilms treated with 2 mg L^{-1} monochloramine. The main loss of physiological activity was observed at the interface between fluid and biofilm.

2.2.4.4 Conclusion

We have demonstrated in Chapter 2 that in presence of different detergent concentrations more EPS remained with *P. putida* WM than with *P. putida* DSMZ, while at the highest concentration, all cells were removed with the detergent. However, the reference strain appeared to withstand detergent stresses much better because more cells remained metabolically active after the treatment.

Therefore, experiments which only consider the usage of the CV assay for testing the anti-biofilm effect of an active agent are not insufficient because they only covering the effect upon biomass reduction. Combinations of assays covering both biomass and viability should be conducted to really define the anti-biofilm effect. Because biofilms are aggregations of cells and organic matter (biomass), a potent anti-biofilm agent removes most or all EPS and inhibits cell activity to reduce the probability for recolonization.

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2.2.5 The effect of different detergent components on biofilm removal

2.2.5.1 Introduction

The goal of this study was to determine, which of the detergent component has the main activity in biofilm removal. The tested standardized washing detergent IEC-A* consists of three components: base, bleach and bleach activator. So far, it is known that bleach has an antimicrobial activity (McDonnell and Russell 1999). However, it was tested, if also the other components of the standard detergent have an effect on biofilm removal.

2.2.5.2 Material and Methods

The organisms

The organisms for testing the removal efficiency of the single components of a standardized washing detergent were *Escherichia coli* PHL628 (curli) an internal reference strain with good biofilm performance and *Pseudomonas fluorescens* (washing machine isolate). A pipette tip-full (ca. 10 µl) of both cryogenic cultures were grown overnight in 8 mL tryptic soy broth (TSB). Three mL of the bacterial suspension was transferred into 300 mL TSB (*E.coli* PHL628: OD_{600}: 0.04, *P. fluorescens*: 0.024). One hundred µL of the diluted bacteria were transferred into sterile 96-well plates (TPP) and incubated (30°C, 100 rpm, 24 h). For each organism two well plates were prepared for the detergent test and a third one served as biofilm control.

After 24 h the suspension (100 µL) was collected and transferred into fresh 96-well plates. The suspension was measured with the platereader (ELx800, BioTek Instruments, Lucerne, Switzerland) at 595 nm.

Washing detergent components

The standard washing detergent (IEC-A*) is composed of 5.39 g L^{-1} IEC-A base, 2.1 g L^{-1} sodium perborate and 0.14 g L^{-1} TAED. The IEC-A* and each single component of the washing detergent were diluted separately in 1 L of cold tap water and diluted in 10-fold steps from 100% to 0.0001%. The well plates were incubated at 30°C for 30 min at 100 rpm. The detergent components were discarded and the biofilms were washed five times

with 300 µL water and air-dried. The biofilms were stained with 0.1% CV and quantified with DMSO according to the previously described protocol. The experiments were conducted in duplicates.

Data analysis

One plate with biofilms was used as biofilm control and the average amount of biofilm was calculated. One plate was filled with different concentrations of detergent and detergent components and served as blank. Because there was no difference in optical density of the detergent and the detergent components, the average of all wells was taken as reference (blank). The average blank was subtracted from each measured biofilm value and from the average biofilm control.

2.2.5.3 Results and Discussion

The three components of the washing detergents contribute differently to the removal of biofilms of *E. coli* PHL628 and *P. fluorescens* (Figure S2.4). Biofilms of *E. coli* PHL628 (curli) (Brombacher et al. 2006) built rather dense biofilms (biofilm/suspension ratio: 11.5) than *P. fluorescens* (biofilm/suspension ratio: 1.7). This could explain why the detergents and the detergent components showed a weaker biofilm removal effect on thicker biofilms of *E.coli* PHL628. Complete removal of *E. coli* PHL628 biofilm, was only observed in undiluted detergent solution and in undiluted IEC-A base (Figure S2.4). In case of the the diluted IEC-A* and IEC-A base, a significant reduction of biofilm in comparison to the untreated control was observed. TAED and Na-perborate did not affect biofilm at all.

In *P. fluorescens* only the tap water treatment (0 % detergent or detergent component) did not completely remove the biofilm. In all the other cases, all single components and the IEC-A* detergent reduced biomass compared to the control.

It is well known that bleaches have an antimicrobial activity (Rutala and Weber 1997; Terpstra 1998). Therefore, it was expected that the bleach component would remove more biofilm than the other two components. A reason why the bleach component (Na-perborate) in our study was less effective is that the experiment was performed at a rather low temperature (30°C). For low temperature washing the bleach activator (TAED) is usually added to the washing detergent. TAED activates the perborate producing an active peracetate aninion with bactericidal activity (Sheane 2000). Because the three components

were tested seperately, the lack of TAED during the assay could lead to a lower activity of the bleach component.

In contrast to this, the IEC-A base by itself removed most of the biofilm. It was assumed, that the alkaline pH (pH = 10 ± 1) of the standard detergent mainly contributed to the removal of biofilms. (Table S2.2). However, the pH of the bleach (1.4 g in one liter of water) was also alkaline (pH = 10). Therefore, the high pH could not explain the high removal activity of the base detergent. Analyzing the composition of the IEC-A* (Table S2.3) it was found that the IEC-A base contains anionic (linear sodium alkyl benzene sulfonate (WFK Testgewebe GmbH) as well as non-ioninc tensides (ethoxylated fatty alcohol). The sodium soap is mainly added as anti-foaming agent. One type of a linear sodium alkyl benzene sulfonate with a biofilm dispersing activity was patented by Yu and McCoy (1997). The main sub-component of IEC-A base is zeolite which together with silicates act as water softener reducing the production of Mg-and Ca-salts and therefore, supporting the activity of tensides. The antimicrobial action of tensides is cell membrane solubization (e.g. solubilization of membrane-bound proteins; Filip et al. 1973) while for bleach (e.g. peracetat anion, an oxdidant derived from Na-perborate), the main action is the oxidation of cellular components (Finnegan et al. 2010).

Table S2.2. Measured pH values of the three IEC-A* detergent components

Detergent	**Amount of component dissolved per 1 L water**	**pH**
IEC-A* standard detergent	5.39 g IEC-A base, 1.4 g Na-perborate and 0.21 g TAED	10 (average)
IEC-A base	5.39 g	10
Na-perborate	1.4 g	10
TAED	0.21 g	7.5 (at 30°C)

Table S2.3. Composition of the IEC-A* standard detergent (modified; WFK Testgewebe GmbH).

Ingredients	**Specification**
Linear sodium alkyl benzene sulfonate	8.8 %
Ethoxylated fatty alcohol C12-18 (7 EO)	4.7 %
Sodium soap	3.2 %
Anti foam DC2-4248S	3.9 %
Sodium aluminium silicate zeolite 4A	28.3 %
Sodium carbonate	11.6 %
Sodium salt of a copolymer from acrylic and maleic acid (Sokalan CP5)	2.4 %
Sodium silicate	3.0 %
Carboxymethylcellulose	1.2 %
Dequest 2066	2.8 %
Optical whitener	0.2 %
Sodium sulfate	6.5 %
Protease	0.4 %
Bleach	20 %
TAED	3 %
IEC-A* standard detergent	100 %

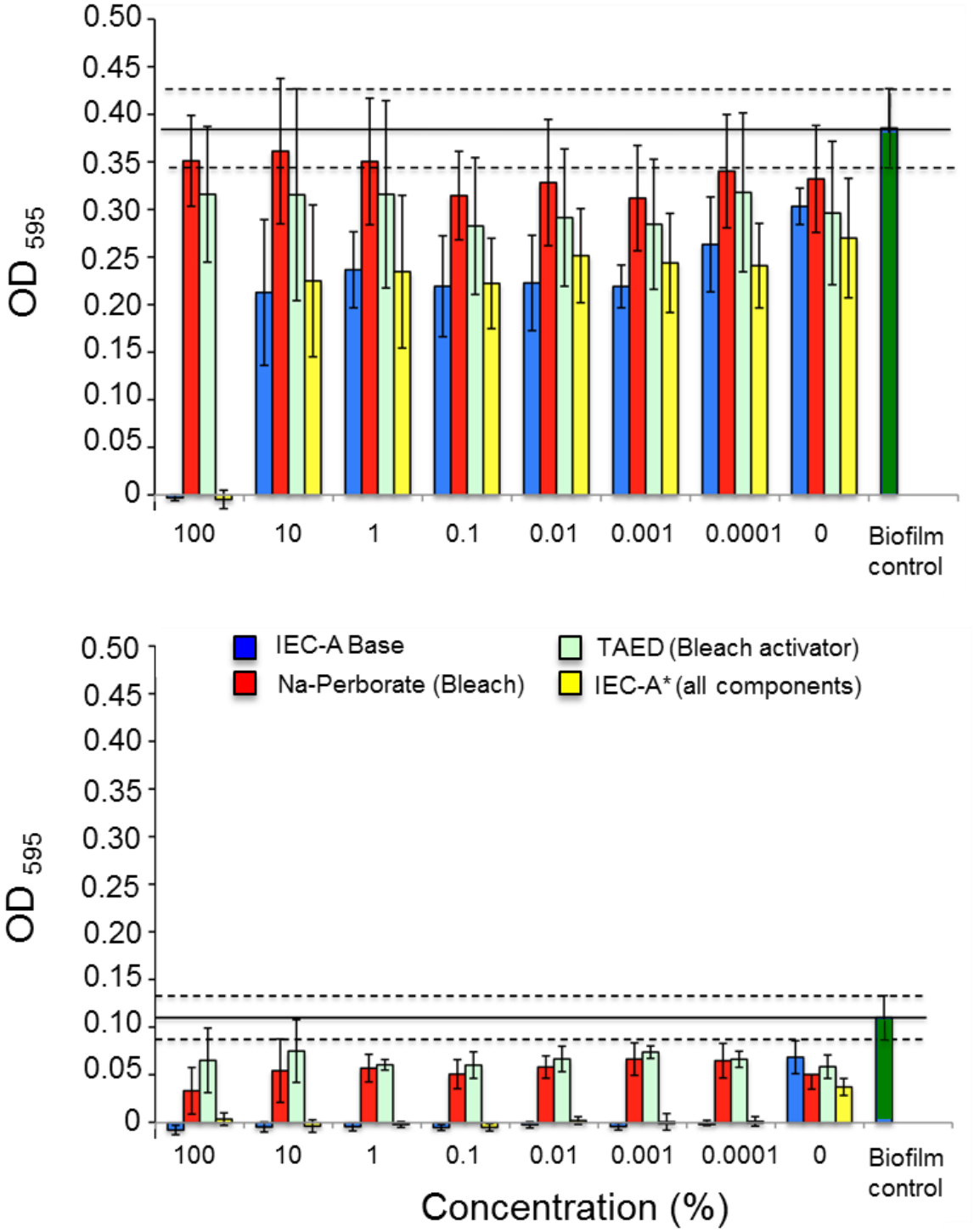

Figure S2.4. Biofilms of *E. coli* PHL628 (upper panel) and *P. fluorescencs* (lower panel) were treated with each single component of the IEC-A* standard detergent with different concentrations at 100 rpm. Line: average count of initial biofilm (control). Dashed line: standard deviation of the biofilm control.

2.2.5.4 Conclusion and Outlook

Biofilms that were 24 h old had the ability to be less affected by the washing detergents especially when they form dense layers (*E. coli*). The main component of the standard detergent IEC-A* is the IEC-A base together with bleach and bleach activator. The main contributor on biofilm removal being the IEC-A base and not the bleach component as primarly expected. The IEC-A contains tensides that are able to physically remove biofilms and destabilize cell membranes, while bleach mainly attack cellular components. However, it could not be excluded that the cell viability was affected because the CV assay only gives information about the total amount of total biomass. The analysis of the effect of each single detergent component on viability would be next logical step, e.g with formazan-based assay such as the INT assay (see 2.2.4) to complement the results of the CV assay.

Further, it would also be interesting to determine, if the Na-perborate in combination with the bleach activator would increase the biofilm removal at 30°C or only affects cell viability.

Reduction of the biofilm matrix decreases the possibility to serve as an anchoring or sheltering site for newly introduced microorganism or as nutrient source. The ideal laundry detergent aims to reduce/remove EPS as well as to inactivate cellular activity.

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3. Biofilm formation of the yeast *Rhodotorula mucilaginosa*: process, repeatability and cell attachment in a continuous biofilm reactor

Jasmin Gattlen[+], Manfred Zinn[+]*, Sébastien Guimond[++], Enrico Körner[++], Caroline Amberg[#] and Laurie Mauclaire[+]

[+]Empa, Swiss Federal Laboratories for Materials Science and Technology, Laboratory for Biomaterials, Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland
[++] Empa, Swiss Federal Laboratories for Materials Science and Technology, Laboratory for Advanced Fibers, Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland,
[#] Empa Testmaterials AG, Moevenstrasse 12, CH-9015 St. Gallen, Switzerland

*Corresponding author.
Mailing address:
Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland.
Telephone: +41 58 765 76 98
Fax: +41 58 764 77 88
E-mail: manfred.zinn@empa.ch

The manuscript was accepted on July 2011 with minor revisions by the Journal "Biofouling: Journal of cell adhesion and biofouling".

My contribution was the cultivation of the yeast biofilm, their analysis and the writing of the manuscript.

Abstract

Yeast biofilms contribute to quality impairment of industrial processes and also play an important role in clinical infections. Little is known about biofilm formation and their treatment. The aim of this study was to establish a multi-layer yeast biofilm model using a modified 3.7 L bench-top bioreactor operated in continuous mode (D =0.12 h^{-1}). Repeatability of biofilm formation was tested by comparing five bioprocesses with *Rhodotorula mucilaginosa,* a strain isolated from washing machines. The amount of biofilm formed after 6 days post inoculation was 83 µg cm^{-2} protein, 197 µg cm^{-2} polysaccharide and 6.9 x 10^6 CFU cm^{-2} on smooth polypropylene surfaces. Roughening the surface doubled the amount of formed biofilm but also increased its spatial variability. Plasma modification of polypropylene significantly reduced the hydrophobicity but did not enhance cell attachment. Finally, the biofilm formed on polypropylene coupons could be used for sanitation studies.

Keywords: yeast, *Rhodotorula mucilaginosa*, model biofilm, test system, bioprocess

3.1 Introduction

The wealth of information on development, structure and impact of bacterial biofilms in different fields such as medicine, biotechnology or ecology is enormous. In comparison to that, the knowledge about fungal biofilms (both yeast and filamentous fungi) is still in its infancy.

Yeast biofilm development is similar to the one identified for bacteria (Harding et al. 2009). The main phases of biofilm formation such as adherence, microcolony formation, maturation, biofilm maintenance and dispersal occur (Harding et al. 2009). The main difference in biofilm development is the morphological transition of some yeast cells during maturation. Dimorphic yeasts, like *C. albicans* and *S. cerevisiae,* attach to the surface and build a monolayer as spherical cells and pseudohyphae during maturation (Vopalenska et al. 2010). *Rhodotorula* sp. also has the ability to form pseudohyphae but their formation has not been reported during biofilm formation. For the examination of cell attachment, biofilm growth and production reactors like perfusion chambers (Palmer 1999), modified Robbins devices (Kharazmi et al. 1999) or rotating disk reactors (Hentzer et al. 2001) are used. These systems focused on bacterial biofilm, whereas studies with yeast cells have hardly been performed (Busscher et al. 1994). From a practical point of view yeast are more complicated to cultivate than bacteria because of a rather slow growth and a higher susceptibility to bacterial (Saithong et al. 2009) or other yeast contaminations (Kronlof and Haikara 1991). Yeast have excellent abilities to grow directly on plastics (Reynolds and Fink 2001) or stainless steel (Brugnoni et al. 2007) but also on bacterial (Jenkinson and Douglas 2002) or fungal biofilms (Webb et al. 2000) as a secondary colonizer.

Biofilm comprising yeast occur not only on implants (Douglas 2002) but also in industrially relevant devices such as photo-processing tanks (Elvers et al. 1998) or food processing plants (Brugnoni et al. 2007) where yeast biofilms have an influence on the quality and taste of the product. But they are also found in domestic environments such as kitchen sponges, dish towels (Rayner et al. 2004) or household washing machines (Gattlen et al. 2010). Biofilms in household washing machines produce malodor in the washing machine and impair its hygienic performance due to increased low-temperature and bleach-free washing (Munk et al. 2001). In industry, biofilms lead to costs of several billion dollars every year due to product losses (Kumar and Anand 1998), reduced heat

transfer (Shi and Zhu 2009), increased fuel consumption (Chambers et al. 2006) and the need for and use of chemical agents for the control and removal of biofilms (Lyon et al. 2008). Besides chemicals (e.g. antimicrobial agents), several mechanical strategies are available to remove biofilms (e.g. ultrasound) (Muller et al. 2007). However, their efficiency (especially of antimicrobial agents) in biofilm removal remains unclear because there are still very few test systems available to evaluate biofilm removal (Hamilton 2002; Pitts et al. 2003; Bloss and Kampf 2004). One reason explaining why biofilm removal cannot be efficiently quantified is the lack for a reference biofilm. Such reference material should represent the system of interest and be produced in a repeatable manner. Short-term studies of antimicrobial and biofilm removal tests can be performed in 96-well plates for bacteria (Pitts et al. 2003) and yeasts (Rambali et al. 2001). Chandra and co-workers (Chandra et al. 2001) produced a 24h-old *C. albicans* model biofilm on prosthesis material cultivated in 12-well tissue culture plates for testing antifungal agents. Ramage and co-workers (Ramage et al. 2001) developed a high throughput 96-well plate system to produce and study *C. albicans* biofilms. However, all these biofilms were cultivated in well plates. The cultivation of biofilms in well plates is limited to young biofilms (24 - 48 h old) because continuous nutrient supply is not possible. Therefore, they are not representative for thicker and/or older (initially mature (Harding et al. 2009) or mature) biofilms as they are typically found in industrial plants or home appliances. An alternative to study initally mature biofilms is the use of bioreactors. This was successfully done for bacterial biofilms using either rotating disk reactors (Pitts et al. 2001) or a reactor developed by the Centers for Disease Control (CDC reactor) (Goeres et al. 2005; Hadi et al. 2010).

Available yeast models that are not used for testing antimicrobial susceptibility focus more on the developmental characteristics of biofilm formation (Ramage et al. 2001). A laminar flow or biofilm bioreactor system is more convenient for the study of initially mature biofilms because flow systems can be adjusted to represent particular physiological conditions (e.g. nutrient limitations, different shear stress, etc.) better than well plates.

A further aspect for the development of a model biofilm is that the resulting biofilm is repeatable and reproducible (e.g. amount of cells, total protein). Repeatable biofilm formation has already been successfully achieved with bacteria grown in rotating disk reactors (Pitts et al. 2001), rotating annular reactors (Chen and Stewart 2000) and in the CDC reactor to evaluate the effects of chemical agents (Goeres et al. 2005; Hadi 2010). First attempts to grow *C. albicans* in a CDC reactor were described by Honraet and co-

workers (Honraet et al. 2005), where the main goal was to test different quantification techniques rather than producing a model biofilm.

However, to our best knowledge yeast model biofilms have not been produced in a comparable reactor system which would allow the reproducible production of biofouled test coupons. Such standardized biofilms could be of use for testing the removal efficiency of cleaning and sanitation (either mechanical or with antimicrobial and chemical agents). Further, they could be applied for testing tolerance towards detergents or antimicrobial agents as well as dosage effect. The potential field of applications could be medical devices but also water pipes (either cooling or water distribution systems) and manufacturing procedures (e.g. plate heat exchanger of pasteurizers for diary processing).

The goal of this study was to establish a model yeast biofilm to be used as reference for testing removal efficiency of household washing machines (Gattlen et al. 2010). The yeast *R. mucilaginosa* that was originally isolated from household washing machines was chosen as model organism. *R. mucilaginosa* was grown in a modified bench-top reactor to firstly determine the cultivation conditions in minimal medium for the optimal production of a multi-layered yeast biofilm and secondly, to assess repeatable biofilm formation on polypropylene coupons with different surface characteristics (smooth and rough, as well as plasma treated).

3.2 Materials and Methods

3.2.1 Bioreactor set-up

For the experiment a modified 3.7 L bench-top bioreactor (KLF2000, Bioengineering AG, Wald, Switzerland) was used (Figure 3.1). A stainless steel cylinder (height: 20 cm, maximal diameter: 6 cm) designed to hold six removable coupon holders (stainless steel) with space for 20 test coupons was mounted on the stirrer axis replacing the stirrer blades. The coupons were immersed in 70% ethanol (EtOH) and sonified in a water bath for at least 10 min before mounting into the metal holders for chemically cleaning of the coupon surfaces. In order not to modify the surface ethanol was chosen for cleaning. Autoclaving sterilizes the surface but is not able to remove e.g. grease. The test coupons were used only once. The pH probe (RedCap 405-60-T-S7/120/9848, Mettler Toledo, Greifensee, Switzerland) was calibrated with two reference solutions with pH = 4 and pH = 7

(BioChemika). An external aeration loop was connected to the reactor to avoid bubble formation that could result in additional shear force and remove biofilm from test coupons. A trap column for liquids was connected between the 50 L medium bag and the reactor to prevent back contamination of the medium bag.

The biofilm reactor was filled with 2.5 L of 30% Sabouraud dextrose broth (SDB, pH = 5.6) to control and slow down cell growth as well as to adjust the cells to a poor nutritional environment as will follow during continuous cultivation. The medium was autoclaved (30 min at 121°C). The external aeration bottle filled with ca. 500 mL of 30% SDB (Figure 3.1, K), the whole tubing system as well as the glass columns (see Figure 3.1, C and D) were autoclaved separately. The aeration of the biofilm reactor via external aeration loop (Figure 3.1, K) was initiated ca. 12 - 15 h prior to inoculation to stabilize the system and ensure sufficient dissolved oxygen tension during inoculation. The cyclic flow in the aeration loop was maintained by a peristaltic pump (Periplex, Bioengineering AG) (Figure 3.1, G) running at maximum speed and an overpressure triggered by the aeration of filtered air via a ventilation frit (Figure 3.1, M).

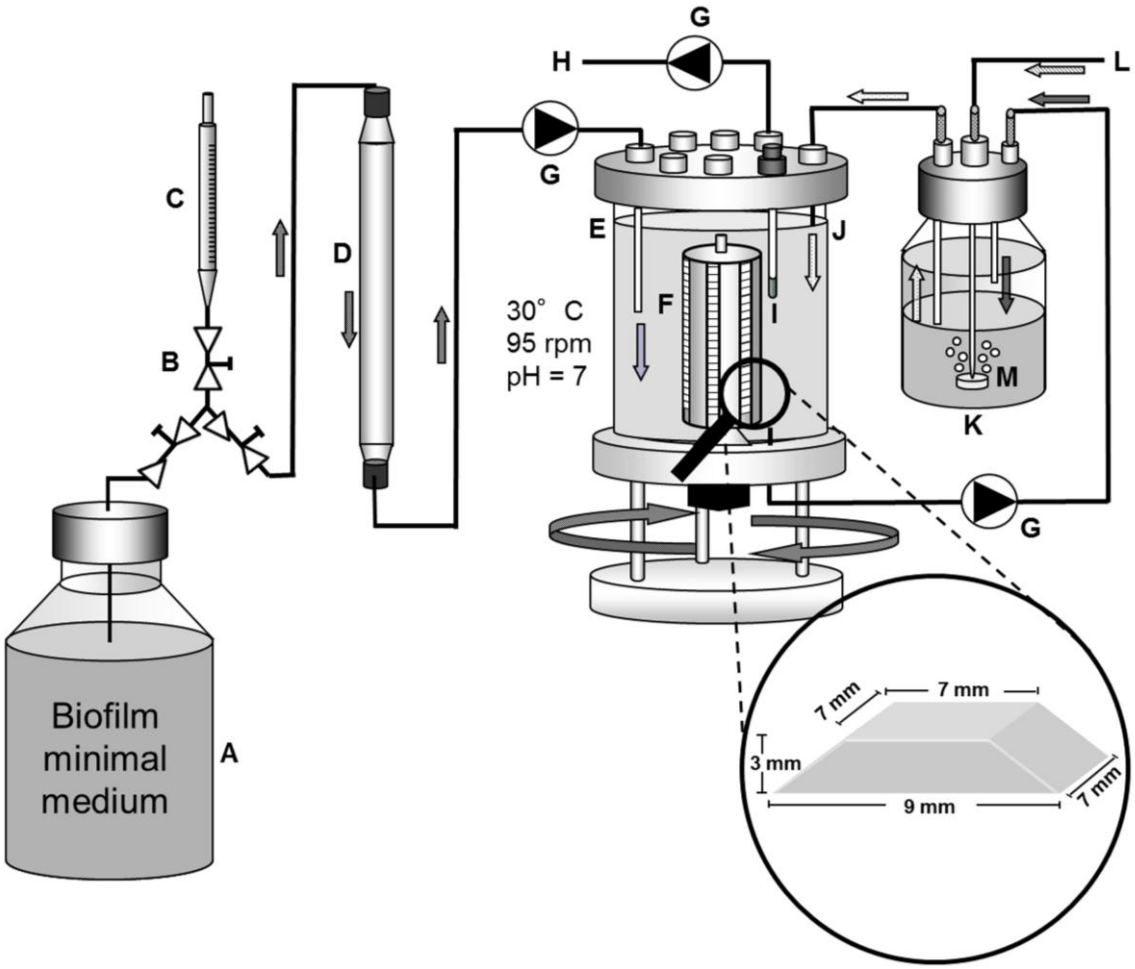

Figure 3.1. Set up of reactor system for biofilm formation. A: medium reservoir (50 L) with biofilm minimal medium, B: valve, C: glass burette for flow measurements, D: glass column for prevention of back contamination, E: submerged inlet tube for biofilm minimal medium feed, F: rotating cylinder with coupons, G: peristaltic pumps, H: outlet waste, I: pH meter, J: inlet of aerated medium, K: aeration bottle, L: inlet for pressurized air, M: ventilation frit, magnification of the trapezoid PP coupons. Biofilm formation of coupon occurred only on top surface (7 x 7 mm).

3.2.2 Characterization of test coupon material

PP composition and surface roughness. The supporting material for biofilm formation was white polypropylene (PP) reinforced with glass fibers (Lot Nr.: PP Miele Granulate Hostacom EKG W92535, Germany). The PP plates were cut to trapezoid coupons (top surface 7 mm x 7 mm, bottom surface: 7 mm x 9 mm, thickness: 3 mm) (Figure 3.1) and either used unchanged or with a roughened top surface with a sandpaper/abrasive paper (150 grains cm^{-2}), perpendicular to the direction of rotation in the reactor. Alternatively, a set of dye-casted PP (dcPP) with a defined roughness (roughness 24, defined according to

VDI 3400; Treff AG, Degersheim, Switzerland) was used to examine the influence of the coupon material on biofilm formation. The composition of the dcPP was similar to the PP provided by Miele, however, without glass-fibres and zinc oxide (white color) as additives.

Plasma treatment of coupons. To reduce the hydrophobic nature of the dcPP coupons, they were plasma activated or plasma coated using the following process gases and gas mixtures: Ar/O_2, N_2, NH_3/C_2H_4 (ratios 1:1 and 2:1) and CO_2/C_2H_4 (ratios 2:1 and 6:1). The C_2H_4 based gas mixtures led to the deposition of plasma polymer thin films where either N- or O-containing functional groups were embedded. The functional groups based on N_2 and Ar/O_2 were directly grafted onto the PP surface. The exact procedure was carried out as described elsewhere (Hegemann et al. 2007; Hossain et al. 2007; Koerner et al. 2009). Prior to reactor experiments the coatings were tested for heat stability (121°C, in presence of culture broth). The composition of the coupon surface was characterized by X-ray photoelectron spectroscopy (XPS; PHI 5600 spectrometer, USA, n = 1) and static contact angle measurements using a droplet of distilled water (~5 µL) (n = 3). A set of 18 coated coupons (3 coupons for each plasma coating condition) was autoclaved in the presence of 30% SDB to simulate the conditions within the reactor during medium sterilization and to check the influence of the culture medium on the plasma-coating. The samples were air-dried under laminar flow in the sterile bench for 2.5 h and contact angles were measured (Krüss G10 apparatus, Hamburg, Germany). In order to evaluate the stability of the functionalization after two days, the samples were dipped for 30 s in nanopure water, dried for 2.5 h and water contact angles were measured again. Freshly coated coupons were used for cultivation experiments.

3.2.3 Cultivation of the yeast *Rhodotorula mucilaginosa*

Rhodotorula mucilaginosa, a pigmented yeast typically living in terrestrial and aqueous habitats, was isolated from a household washing machine (Gattlen et al. 2010) and was used throughout all experiments.

Preparation of frozen stocks. Since frozen stocks are a potential source of variability, the preparation of the stocks was performed with specical care. A colony of *R. mucilaginosa* grown on Sabouraud 4% glucose agar (SDA) was transferred into 15 mL SDB and incubated for ca. 18 h (30° C, 150 rpm). The culture was used to inoculate a shake flask containing 100 mL SDB. The cells were grown (150 rpm, 30°C) until an OD_{600} of about 0.5 - 1.0 was reached. The culture broth was mixed 1:1 (v v^{-1}) with 30% glycerol and 2 mL

aliquots were prepared. Cells were frozen at -20°C overnight and stored at -80°C until usage.

Preparation of pre-cultures. For the preparation of the bioreactor inoculum one vial with frozen yeast cells (2 mL) was transferred into a baffled shake flask containing 150 mL SDB supplemented with anhydrous ampicillin (final concentration: 50 µg mL^{-1}) and chloramphenicol (final concentration: 500 µg mL^{-1}). Antibiotics were used to prevent contaminations with bacteria. Cells were incubated at 30°C, 150 rpm for ca. 24 h until reaching an optical density of 2.6 ± 0.1.

Inoculation of the bioreactor. Cells reaching the late exponential phase were inoculated into the biofilm reactor at 30°C and a cylinder rotation of 95 rpm. Cell growth was followed by measurements of OD_{600}. When the maximal growth rate μ_{max} (0.23 - 0.27 h^{-1}) was reached the washout of cells in suspension was initiated. In order to wash out the non-adhering cells continuous cultivation was started with an initial dilution rate of ca. 0.52 h^{-1} with biofilm minimal medium. The biofilm minimal medium (pH = 7) for continuous cultivation consisted of 1 g L^{-1} 3-(N-morpholino)propanesulfonic acid (MOPS), 1.1 g L^{-1} $(NH_4)_2SO_4$, 0.15 g L^{-1} KH_2PO_4, 0.25 g L^{-1} $MgSO_4$ x 7 H_2O, 0.1 g L^{-1} $FeSO_4$ x 7 H_2O, 0.2 g L^{-1} ethylenediaminetetraacetic acid disodium salt and 1 mL of filter-sterilized (0.22 µm, Milex, Milipore AG, Zug, Switzerland) trace element stock solution (1.5 g L^{-1} $CaCl_2$ x $2H_2O$, 3.96 g L^{-1} $MnCl_2$ x $4H_2O$, 5.62 g L^{-1} $CoSO_4$ x $7H_2O$, 0.34 g L^{-1} $CuCl_2$ x $2H_2O$, 1 g L^{-1} $ZnSO_4$ x $7H_2O$, 1 g L^{-1} MoO_4Na_2 x $2H_2O$, pH = 1) with 4 g L^{-1} glycerol as carbon source. All chemicals (Sigma-Aldrich, Buchs, Switzerland) except for the micronutrient solution had been autoclaved prior to filter-sterilization (0.45 µm + 0.2 µm; Sartorius) to minimize risk of contamination. In previous experiments it has been observed that filter-sterilization was not sufficient to remove contaminants from the chemicals. The dilution rate was set twice as high as the μ_{max} to wash out non-adhering cells for ca 13 ± 1 h. After the washout the dilution rate was reduced to 0.12 h^{-1} for further cultivation.

3.2.4 Sampling and quantification of formed biofilms

Biofilm formation was assessed on PP coupons mounted in holders on a rotating cylinder (Figure 3.1). For the temporal development of biofilm formation, one holder harbouring 14 test coupons (7 rough and 7 smooth) was harvested after 1, 3, 6, 9, and 13 days post inoculation (p.i.). One smooth and one rough coupon were prepared for microscopic observation by confocal laser scanning microscope (CLSM).

For the repeatability test, all six holders of a bioreactor amounting in n = 16 - 23 smooth and 18 rough coupons were sampled for biofilm quantification on day 6 p.i. The remaining coupons were only used to fill the other positions.

The plasma treated coupons were harvested after 1, 3 and 6 days post inoculation and formed biofilm was quantified (n = 4) as follows:

Sampling of coupons. After removal from the holder unit, each coupon was briefly submerged into sterile 0.9% NaCl solution to remove loosely attached cells. Each coupon was then transferred into 5 mL of 0.9% NaCl and treated with ultrasound (sonifier tip, Branson sonifier) at 0°C (10% amplitude, 30 s with alternating 1 s pulse on and 1 s pulse off) to detach the cells from the coupon. The suspensions were stored at 4°C for maximal four hours due to the large amount of samples that needed to be sonified. The samples were vortexed before subsampling for further analysis.

Optical density. One mL of cell suspension derived from sonified biofilms was measured with a spectrophotometer (Spectronic® Genesys™ 6, UV-visible spectrophotometer, Thermo Electron Schweiz AG, Allschwil, Switzerland) at 600 nm.

Polysaccharide quantification (based on Dubois et al. 1956). Because the main constituents of the EPS matrix are polysaccharides the total polysaccharide amount was quantified (Sutherland 2001; Flemming and Wingender 2010). One mL of cell suspension was taken, 25 µL of 80% (v v^{-1}) phenol dissolved in distilled water were added and the sample was vortexed. Subsequently, 2.5 mL of 98% sulphuric acid (Merck, Zug, Switzerland) were added within 20 - 30 s in the center of the solution to ensure a perfect mixing, vortexed for 1 min, cooled down at room temperature for 10 min, vortexed again and finally incubated in the water bath at 26 ± 1°C for 20 min. Before reading the light absorption at 485 nm, the samples were vortexed again. The standard curve was prepared with D(+)-glucose dissolved in distilled water (0 - 35 µg mL^{-1}). Samples containing sugar concentrations above 35 µg mL^{-1} glucose equivalents were diluted with 0.9% NaCl and re-analysed.

*Protein quantification***.** Proteins are a large component of the microbial cell and also found in the matrix consisting of exopolymeric substances (EPS) (Sutherland 2001), therefore the total amount of protein was analysed. For the quantification of the total protein, the micro BCA protein assay kit (Thermo Scientific, Rockford Illinois, U.S.A.), based on the biuret reaction, was applied according to the instruction of the manufacturer. The standard curve was prepared with BSA (0 - 40 µg mL^{-1}).

Viable cell count. Viable cell counts by colony forming units (CFU) were performed by serial dilutions of the suspension and plating on SDA plates. The plates were incubated for ca. 2 days at 30°C before counting.

3.2.5 Confocal laser scanning microscopy

Sampled coupons were placed on wet paper and kept under humid atmosphere at 4°C for no longer than 5 hours until staining. The coupons were stained for 30 min in the dark with 100 µL of a mixture of 0.1 M Tris buffer (pH = 7.5) and Syto BC (Molecular Probes, Invitrogen, Lucerne, Switzerland, final concentration: 0.5 µM) for staining cell DNA. Concanavalin Alexa-633 (Molecular Probes, Invitrogen, final concentration: 0.1 mg mL^{-1}) was used for staining lectins of the exopolymeric substances sugar residues of the biofilm matrix.

The cells were examined with a confocal laser scanning microscope (Axioplan 2 Imaging LSM 510, Zeiss) at wavelength of 488 and 632 nm for Syto BC and ConcanavalinAlexa-633, respectively. The micrographs were recorded and analyzed with the LSM Image examiner (Zeiss, version 4.0.0.2).

3.2.6 Statistical analysis of samples

In general, the mean values and standard deviations for OD_{600}, polysaccharide, protein and viable cell counts were determined for each sampling day. An exception was the first experiment of the repeatability tests where neither cell number nor polysaccharide quantification was done.

3.2.7 Determination of the repeatability of the bioprocess

Spatial variability within the bioreactor. Data sets for each parameter of all five experiments with smooth and rough coupons were checked for normal distribution using Kolmogorov-Smirnov ($\alpha = 0.05$). It is essential for ANOVA-2 analysis that the sample size for each reactor experiment is the same. In case a coupon was lost during harvesting and consequently no data could be obtained, the mean of the samples with the same position on other holders in the reactor was taken as a value. However, this procedure had to be done only in the case of three coupons, two smooth and one rough.

To determine the homogeneity of the growth conditions along the vertical axis, one holder of each reactor experiment was analysed from top to bottom (position 4 to 18) for smooth and rough coupons (n = 3 - 7 and 3, respectively). The presence of a vertical gradient was tested using the linear regression model ($\alpha = 0.05$). For regression analysis outliers were determined applying the Grubbs test.

Homogeneity of growth conditions within bioreactors was analyzed for five bioprocesses using two-way analysis of variance without repetition (ANOVA-2, $\alpha = 0.05$). Eventually, $\log_{10}$ transformation was applied to achieve normal distribution of the parameters. The total variability was split into the three parameters: vertical positions, horizontal positions and residual error that includes undefined parameters such as handling or cultivation. For the analysis of the vertical position and for the horizontal position three times six coupons were evaluated.

Repeatability of biofilm formation. To test repeatability of biofilm formation five independent reactor experiments with *R. mucilaginosa* were conducted. The biofilm samples were analyzed as previously described. ANOVA-2 with repetition was performed in order to determine the source of variation ($\alpha = 0.05$, n = 18). The total variability was split into "position" and "repeatability", "interaction" and residual error. Missing values (n = 2 per analysis) were replaced by the mean of the values measured at the same position on the five remaining holders. Levene test was used to test the homoscedasticity of each single reactor experiment. In the case of variance equality one-way ANOVA (ANOVA-1) was used to test average equality of each repetition. Under unequal variance conditions the results of the Brown-Forsythe test was considered.

3.3 Results and Discussion

3.3.1 Temporal development of biofilm formation and influence of roughness

Temporal development of the yeast biofilms on smooth and rough PP surfaces was determined over a period of two weeks. In general as observed on CLSM micrographs (Figure 3.2) *R. mucilaginosa* colonized the rougher test coupons significantly better, which is in accordance with previous studies that showed that microbial cells prefer rough surfaces to attach to (Quirynen1991; Muller et al. 2007) because cracks provide a protection from the shear forces (Zottola and Sasahara 1994; Palmer et al. 2007). The

microscopic observations were confirmed by biofilm quantification (Figure 3.3). The number of living cells was significantly lower on smooth coupons compared to rough ones (6×10^6 versus 4×10^7 CFU cm^{-2}, respectively) and did not change significantly over the cultivation period (Figure 3.3a). The decrease of cell number and protein on the rough coupons observed on day 3 p.i. was due to sampling errors (i.e. additional immersion of the coupons into the cultivation medium as the coupon holder was blocked during sampling). Protein content on smooth coupons remained stable over the entire cultivation period, whereas the median increased regularly on rough coupons together with the variability (Figure 3.3b). The total amount of polysaccharides increased regularly together with the variability between samples. This increase was more marked for the rough coupons (Figure 3.3c). It has also been noted that after day 6 p.i., when stopping the rotation of the bioreactor for harvesting the coupons, parts of the biofilm detached from the rough coupons on the different holders.

In order to achieve reproducible biofilm coverage on coupons, random events like erosion or sloughing should be limited. In our experiments sloughing was observed when the biofilm thickness increased and led to a higher resistance of the flow. This is a random event, which creates heterogeneity within the biofilm and does not lead to a reproducible biofilm (Lewandowski et al. 2004). Therefore, it was important to define the time period to harvest the produced biofilm as long as the biofilm did not start to detach. Figure 3.3 showed that 3 days p.i. and 6 days p.i. for rough and smooth coupons, respectively. We rather chose to work with smooth coupons because the process of roughening the surface increases variability of the surface material. Prolongation of the bioprocess up to 6 days p.i. on smooth coupons offered also the possibility to study the different phases of biofilm development. Taking all of this into account, we decided to grow biofilms on smooth coupons for no longer than 6 days p.i. in the following experiments.

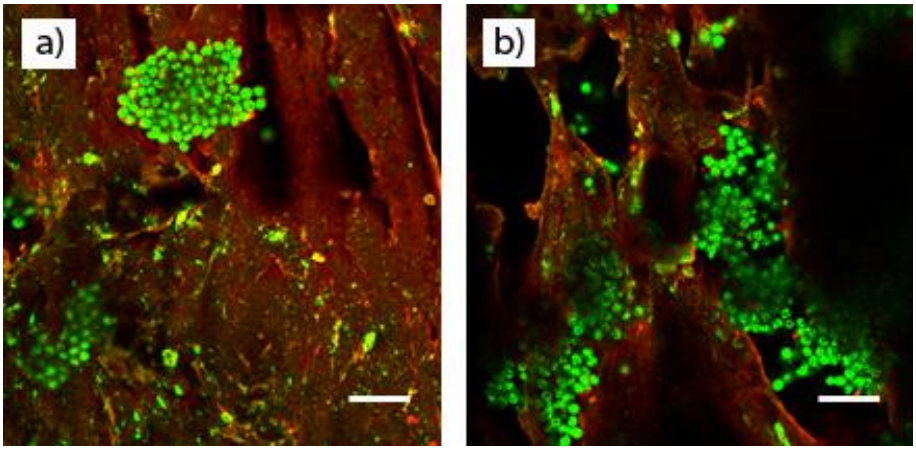

Figure 3.2. Confocal laser scanning microscopy micrographs of one-day old biofilms on a) smooth and b) rough coupons. Two types of staining were used for EPS (ConcanavalinA, red) and cellular DNA (Syto BC, green). The dark red signal is originating from polypropylene. Most of the cells were gathered around little scratches on the surface that protect them from shear forces. The scale bar represents 20 µm.

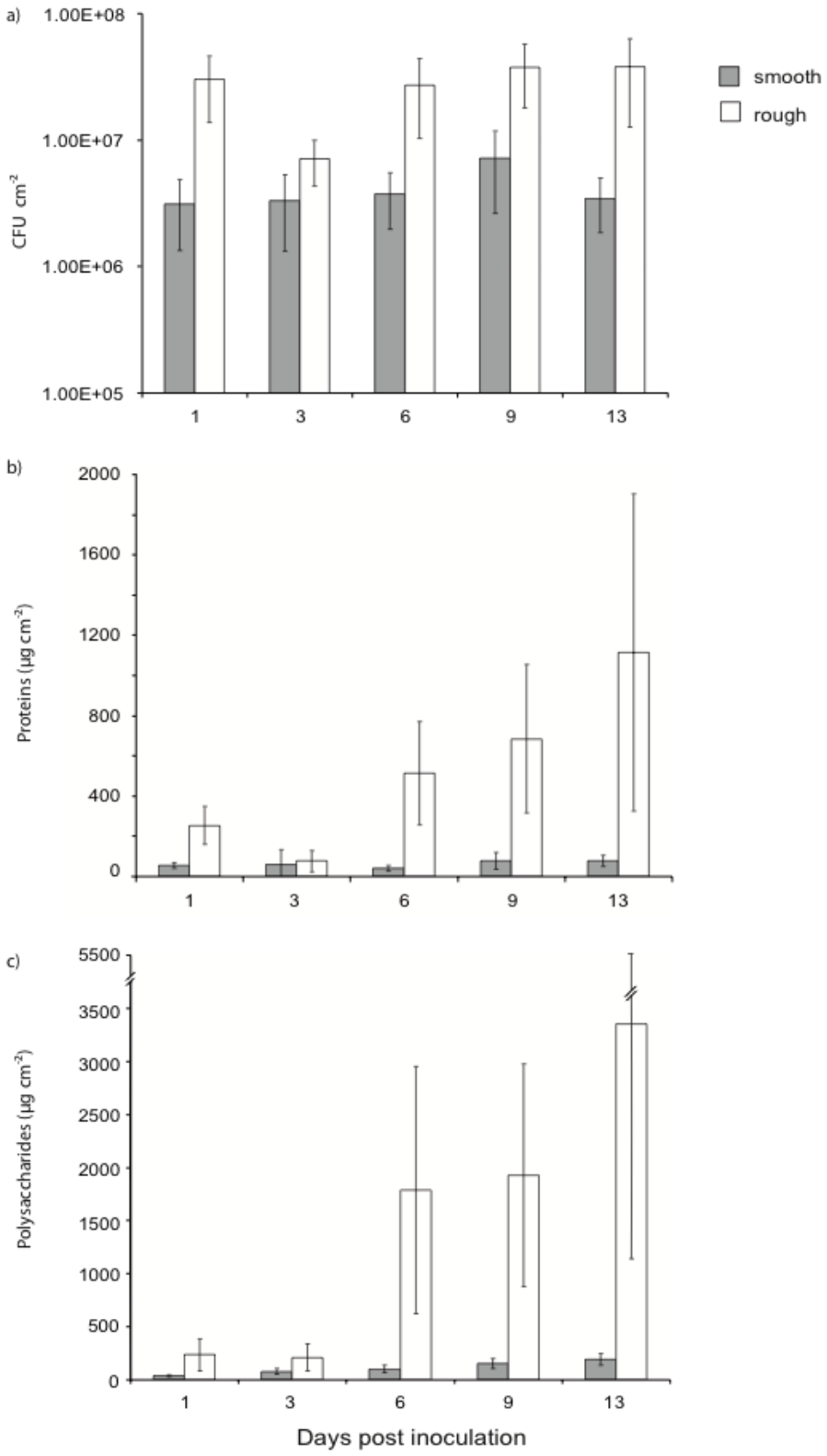

Figure 3.3. Time course experiment with *Rhodotorula mucilaginosa* over 13 days post inoculation. Mean values and standard deviation of a) the colony forming units (CFU) per cm^2, b) amount of proteins per cm^2, and c) amount of polysaccharides per cm^2. Gray bars: smooth coupons, white bars: rough coupons.

3.3.2 Spatial variability within the reactor

Biofilms produced in five independent reactor experiments were harvested, sampled, and quantified on day 6 p.i. For all tested parameters in all experiments the largest source of variability was the vertical position (i.e. variability between the coupons located on the same sample holder) 24 - 83% within the reactor compared to the horizontal position (i.e. variability among the 6 sample holders) 1 - 34% in a reactor (Table 3.1). However, the residual error contributed significantly to the overall variability (10 - 74%).

Table 3.1. Summary of results for biofilm accumulation at day 6 post inoculation for smooth coupons and distribution of the source of variance within a reactor experiment.

Parameter	Components of total variability	Exp. 1	Exp. 2	Exp.3	Exp. 4	Exp. 5
OD_{600}	Average value	0.07	0.05	0.06	0.04	0.06
	Standard deviation	0.02	0.03	0.03	0.02	0.02
Components of total variability	Vertical (%)	42.7	30.0	82.6	27.1	25.1
	Horizontal (%)	15.9	4.7	7.9	1.3	1.1
	Residual error (%)	41.4	65.4	9.5	71.6	73.9
Protein	Average value (µg cm^{-2})	85.0	87.1.	77.7	57.0	82.6
	Standard deviation (µg cm^{-2})	23.1	43.2	36.9	38.1	24.7
Components of total variability	Vertical (%)	35.5	25.1	73.7	38.9	24.1
	Horizontal (%)	10.0	3.7	5.3	2.4	29.4
	Residual error (%)	54.5	71.2	21.1	58.7	46.5
Polysaccharide	Average value (µg cm^{-2})	n. d.	253.9	189.9	127.4	166.1
	Standard deviation (µg cm^{-2})	n. d.	175.5	99.1	38.7	69.2
Components of total variability	Vertical (%)	n. d.	25.6	43.3	27.1	30.2
	Horizontal (%)	n. d.	4.0	8.0	6.3	30.4
	Residual error (%)	n. d.	70.4	48.7	66.6	30.4
CFU	Average value (CFU cm^{-2})	n. d.	1.0E+07	5.1E+06	4.8E+06	6.1+06
	Standard deviation (CFU cm^{-2})	n. d.	9.8E+06	3.3E+06	2.7E+06	4.2E+06
Components of total variability	Vertical (%)	n. d.	39.1	25.7	28.7	38.6
	Horizontal (%)	n. d.	5.4	33.9	11.8	2.2
	Residual error (%)	n. d.	55.5	40.4	59.5	59.2

The variance components are shown as percentages of the total variability

n. d.: not determined

The extent of the vertical gradient present in the bioreactor was illustrated for protein content on smooth (Figure 3.4) and rough coupons (see Figure S3.4 supplementary data). Analysis of the biofilms on smooth coupons with linear regression revealed that the vertical gradient was significant ($p < 0.05$) only for the reactor experiment No. 2 for protein (Figure 3.4), optical density and viable cell counts (see supplementary data Figure S3.3). For rough coupons no significant vertical gradients were detected. For the remaining experiments and for the rough coupons vertical gradients were not significant.

The presence of horizontal and vertical gradients with respect to the thickness of formed biofilm was reported for the rotating annular reactor (RAB reactor) (Gjaltema et al. 1994; Neu and Lawrence 1997). By contrast, CDC reactors appeared to depict no significant spatial gradient (Goeres et al. 2005). One possible explanation is that the distance between the three coupons of the CDC reactor was relatively small (ca. 5 cm), whereas in our system the whole length of coupons in a row was 14 cm and in the RAB 10 - 15 cm (Lawrence et al. 2000; Milferstedt et al. 2006). A possibility to prevent sedimentation and the formation of thick biofilm at the bottom of the reactor would be to increase the shear flow or turbulence. In our system the mixing was performed by the rotation of the cylinder holding the test coupons and by the flow caused by the external aeration loop.

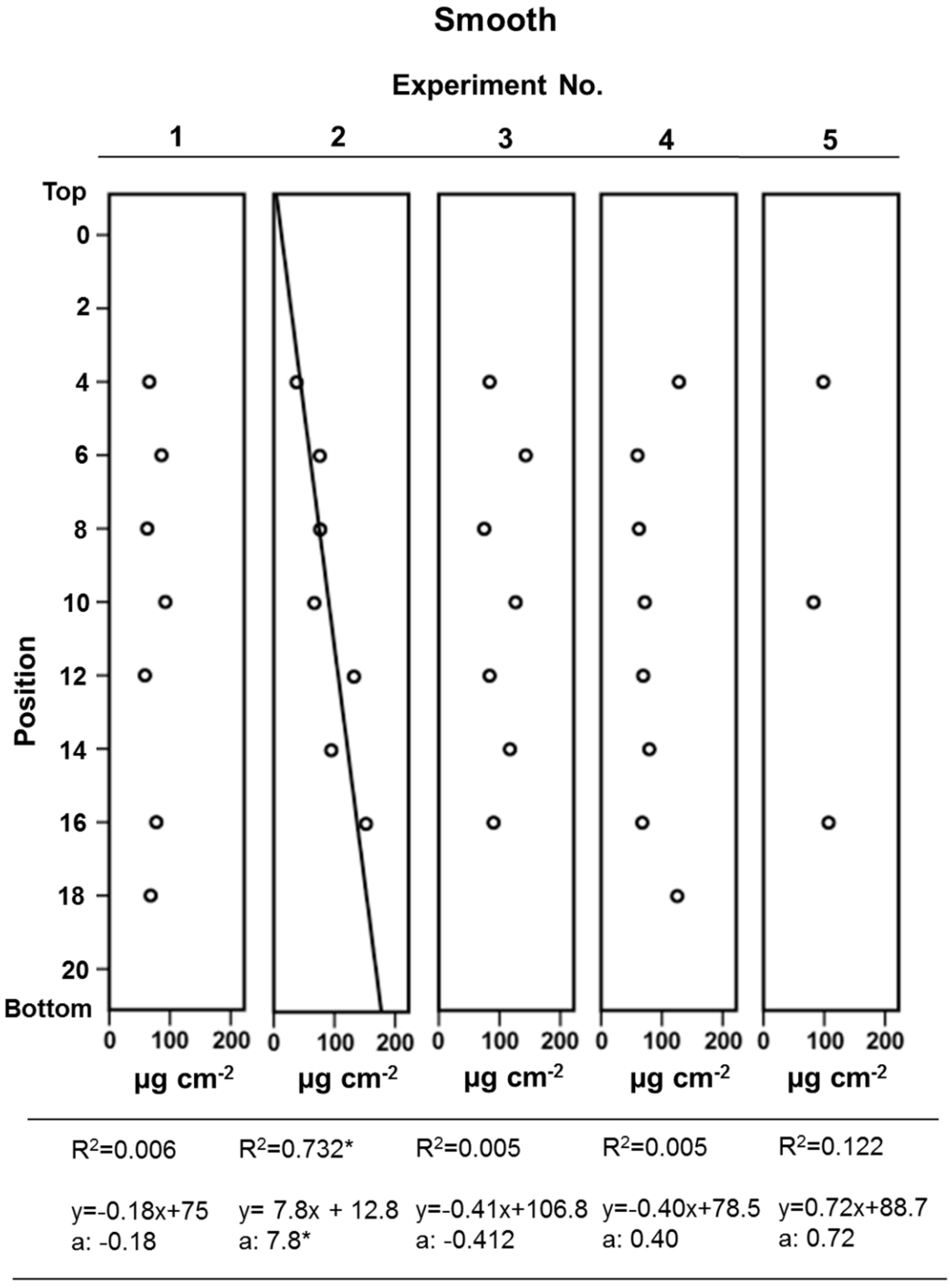

Figure 3.4. Vertical distribution from top to bottom of the protein amount for the five independent experiments on smooth coupons. Linear regression models with significance of R^2 and slope are indicated below ($\alpha = 0.05$), (*): significant.

3.3.3 Repeatability of the bioprocess

The amount of biofilm on smooth coupons of each reactor experiment was quantified and displayed in Figure 3.5. The biofilm characterized by quantification of the amount of protein and polysaccharides, viable cell count, and optical density was similar from one experiment to another for both smooth and rough coupons. The medians of the tested parameters (OD_{600}, protein and polysaccharide amount and viable cell count) varied between reactor experiments, but the values for the n = 4 - 5 experiments were always overlapping, none being completely different from another experiment. Also the ranges (minimal to maximal) of the values for the single reactor experiments were similar except for reactor No. 3 which also showed the largest variability between the coupons.

Taking all reactor experiments (n = 5) only for smooth coupons into account, the mean of the formed biofilm of each reactor experiment was statistically different from one run to another (e.g. ANOVA-1 for polysaccharide amount p-value: 0.008) except for protein amount (p-value: 0.066). The difference from the minimal mean to the maximal mean on smooth coupons of the five independent reactor experiments was 45% for optical density, 38% for protein, 52% for polysaccharide and 50% for CFU. The main reactor experiment which showed the largest difference is reactor experiment No. 4 (Figure 3.5). For protein and optical density the differences of the remaining reactor experiments did not exceed 20%, while for polysaccharide and CFU the minimal difference was still around 35% and 42%, respectively. The larger differences for polysaccharide and CFU could be mainly the results of several required handling and dilution steps of the analytical method.

Interestingly, distribution of the medians and the single values were larger for rough coupons than for the smooth coupons, indicating heterogeneity of biofilm formation probably due to sloughing (Figure 3.6). It can also be assumed that due to the increased surface area (Katsikogianni and Missirlis 2004; Palmer et al. 2007) and attachment possibilities the cells could establish biofilm faster on rough coupons. This could consequently lead to earlier sloughing events. To compare the mean of the smooth and the rough coupons a T-test was conducted. It revealed that the biofilm was statistically different ($p < 0.000$) for smooth and rough coupons.

The variability was analyzed using ANOVA-2 with “repeatability” (between experiments) and “position” (in-between experiments) as sources of variability (Table 3.2). A main source of variability was “repeatability” ranging from 13 - 21% (smooth) and 12 - 34% (rough) of the total variability. The parameter ”position” had less influence on the total

variability ranging from 3 - 4% (smooth) and 2 - 7% (rough) of the total variability. However, the main source of variability was a "residual error" that could be due to other undefined parameters such as harvesting, handling or the method of measurement that represented 71 - 75% and 54 - 74% for smooth and rough coupons, respectively.

Pitts and co-workers (Pitts et al. 2001) reported that the among-experiment variability contributed the least to the total variability. In the experiments without chlorine treatment the within-reactor variability was about 60 and 73%, respectively, while the among-reactor variability was 40 and 27% (Pitts et al. 2001). In our case we observed that most of the variation from one to another reactor experiment comes from the remaining variability (residual error)/parameters (e.g. handling). Therefore finding the most appropriate method for biofilm quantification resulting in smaller residual errors is crucial for testing repeatability.

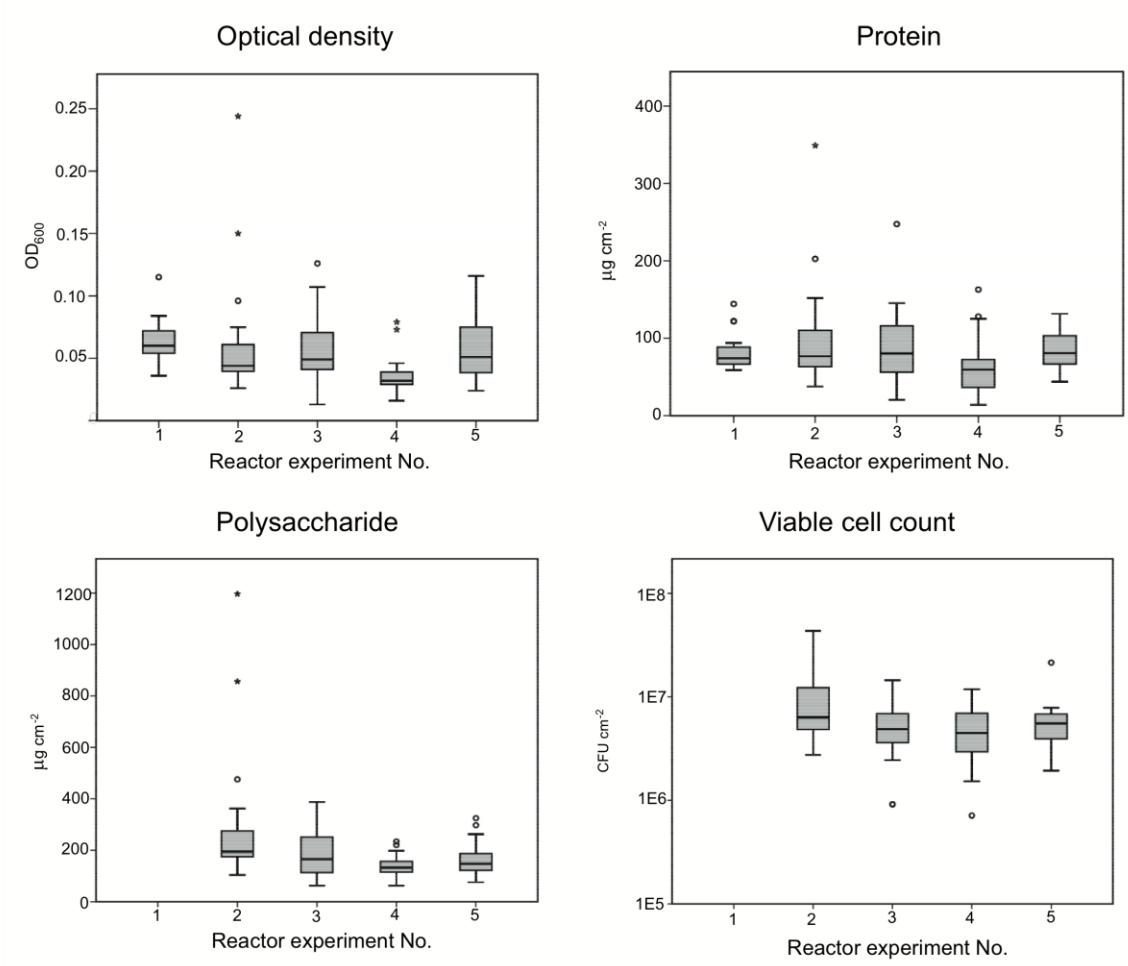

Figure 3.5. Box plot analysis of biofilm formed on smooth coupons. For 4 - 5 independent experiments OD_{600}, protein, polysaccharide, and viable cell counts were quantified. Whiskers: maximal and minimal values, bold line: median, white circles: outlier, stars: extreme values.

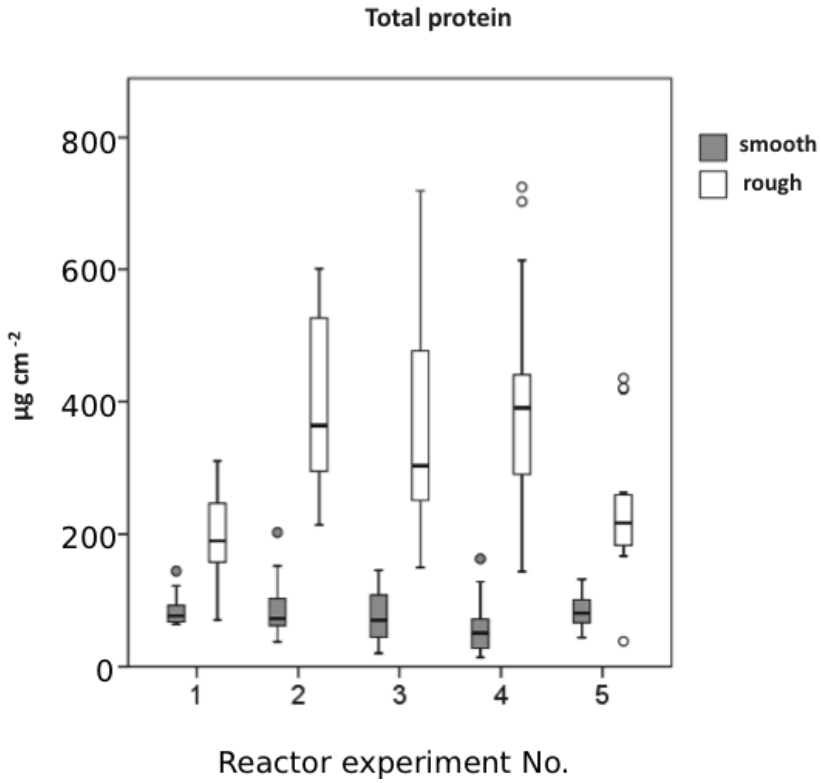

Figure 3.6. Box plot of total protein content of formed biofilm. a) smooth (grey bars) and b) rough PP (white bars) coupons (µg cm^{-2}) were used for five independent experiments with (n = 18 – 23) coupons per reactor. Whiskers: maximal and minimal values, bold line: median, white circles: outlier, stars: extreme values.

Table 3.2. ANOVA-2 of results for biofilm accumulation at day 6 post inoculation for smooth and rough coupons. All parameters were log_{10} transformed.

	OD_{600}	**Proteins**	**Polysaccharides**	**CFU**
Smooth coupons				
Repeatability (%)	21.2	17.9	18.9	13.2
Position (%)	2.6	4.4	4.4	9.2
Interaction (%)	3.7	2.2	5.9	2.9
Residual error (%) (handling etc.)	72.5	75.5	70.9	74.6
Rough coupons				
Repeatability (%)	12.1	34.3	18.8	21.6
Position (%)	2.2	2.9	6.7	4.1
Interaction (%)	12.2	8.4	13.3	2.4
Residual error (%) (handling etc.)	73.6	54.4	61.2	71.9

The variance components are shown as percentages of the total variability

3.3.4 Influence of the supporting material

Wettability and surface composition. Plasma treatments were used to investigate the influence of wettability and surface composition on fouling. Non-treated PP coupons (rough, smooth and dcPP) had water contact angles of ca. 90°. Plasma treatments led to similarly significant increase of hydrophilicity that remained after both autoclaving and rinsing the SDB films (Table 3.3). The coupons were exposed to 30% SDB to simulate the conditions within the reactor as previously described in the *in situ* sterilization protocol. All surfaces contained an elevated number of N and O atoms. The surface composition remained stable after the sterilization process besides a few changes in the amount of O and N atoms (Table 3.4). To evaluate the attachment of cells and further biofilm growth on the plasma treated surfaces, the coupons were harvested after 1, 3 and 6 days. The initial cell attachment as well as the early fouling (day 3 p.i.) were similar for all plasma treated surfaces (Figure 3.7). After 6 days p.i. some differences could be observed, e.g. Ar/O_2 treated coupons being significantly less fouled than CO_2/C_2H_4 plasma treated surfaces (Figure 3.7). NH_3/C_2H_4 based polymers have already been described to enhance cell adhesion due to the high content of amino groups (Truica-Marasescu and Wertheimer 2008). For example, the attachment of mouse fibroblasts was significantly increased on NH_3/C_2H_4 treated poly(L-lactide) material compared to untreated material (Wan et al. 2003). Similarly, oxygen-based functional groups have been reported to enhance cell attachment (Wei et al. 2007) under static or low shear conditions. In our experiments, these two types of plasma-based surface modifications did not enhance the attachment of cells although the surfaces were highly hydrophilic.

The influence of too high shear forces can be excluded because smooth and roughened coupons showed significant biofilm formation under identical growth conditions.

It seems that cell adhesion is enhanced only in the case of mammalian cells, which is in line with other reports where plasma polymerization techniques. In terms of bacterial cells, the plasma-coating technique was applied to incorporate antimicrobials and other toxic compounds to prevent microbial attachment (Jansen and Kohnen 1995; Sen et al. 2009).

Table 3.3. Static water contact angle measurement of plasma treated coupons before and after autoclaving (n = 3).

Treatment	Ratio	Before autoclaving	Autoclaved in SDB	Autoclaved in SDB and rinsed
None	-	~90°	n. d.	n. d.
CO_2/C_2H_4	2:1	55° ± 1°	3° ± 3°	41° ± 4°
CO_2/C_2H_4	6:1	54° ± 3°	flat film	37° ± 3°
NH_3/C_2H_4	1:1	53° ± 2°	flat film	36° ± 9°
NH_3/C_2H_4	2:1	54° ± 2°	flat film	31° ± 5°
Ar /O_2	-	61° ± 2°	31° ± 4°	51° ± 1°
N_2	-	53° ± 2°	16° ± 9°	43° ± 4°

n. d.: not determined

Table 3.4. X-ray photoelectron spectroscopy measurements of differently treated dcPP coupons before and after autoclaving.

Treatment	Before autoclaving Relative atomic composition (%)			After autoclaving [a] Relative atomic composition (%)		
	[C]	[O]	[N]	[C]	[O]	[N]
None	98	2	0	n. d [b]	n. d.	n. d.
CO_2/C_2H_4 (2:1)	79	20	1	75	19	6
CO_2/C_2H_4 (6:1)	78	22	0	76	19	5
NH_3/C_2H_4 (1:1)	70	14	16	70	17	12
NH_3/C_2H_4 (2:1)	72	13	15	71	16	13
Ar/O_2	82	15	3	83	12	5
N_2	75	11	14	80	12	8

[a] In presence of 30% Sabouraud dextrose broth and rinsing with nanopure water.
[b] n. d.: not determined

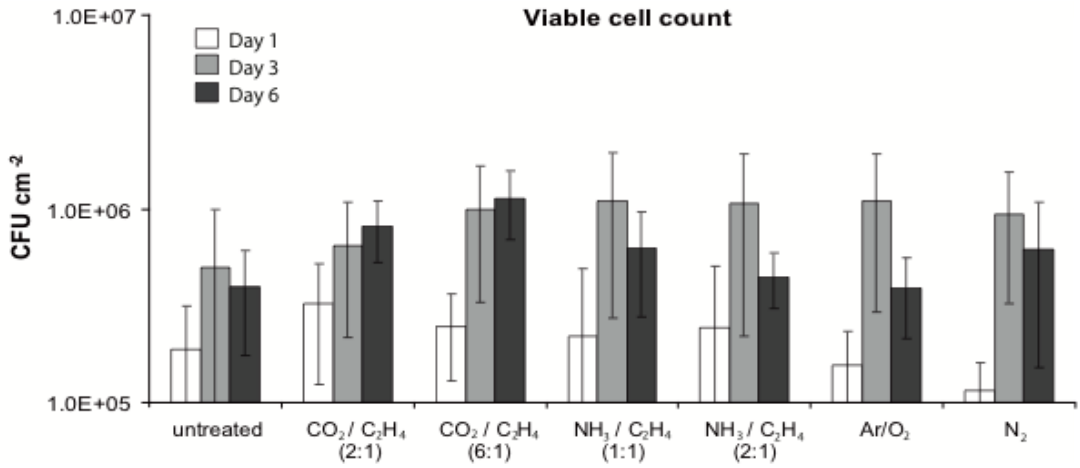

Figure 3.7. Cell attachment and biofilm formation of *Rhodotorula mucilaginosa* on dye-casted rough PP coupons with differently treated plasma-based modifications. Sampling occurred after 1, 3, and 6 days post inoculation with n = 4 coupons per treatment. Shown are the parameters of cell density (CFU cm^{-2}).

3.3.5 Relevance of the study

Standardized biofilms are essential to develop test systems to assess the efficacy of the methods of biofilm removal. Currently, few data about yeast biofilms are available which are mainly about *Candida albicans*. A "new", up-coming opportunistic group of pathogens are *Rhodotorula* species that are causing fungemia in neonates and other immuno-compromised individuals (Duggal et al. 2011).

This is especially true for yeast biofilm for which only few data are available. In this study we presented a reactor system that allowed growth, sampling and quantification of *R. mucilaginosa* biofilms at different stages of development. This bioprocess made use of a commercially available laboratory fermenter with a custom-made rotating cylinder harbouring holders for test coupons. Our bioreactor combined features of the CDC biofilm reactor (Donlan et al. 2002; Goeres et al. 2005) and the RAB reactor (Lawrence et al. 2000) and presented distinct advantages over other systems such as continuous exchange of growth medium or the possibility to conduct *in situ* sterilization i.e. the cultivation medium together with the test coupons can be sterilized within the reactor. *In situ* sterilization of the medium reduced the risk of contamination. Although the entire CDC reactor can be autoclaved it has the disadvantage that the culture medium has to be autoclaved separately and pumped into the reactor afterwards. Due to integrated

temperature and pH controls the overall handling of the system is facilitated and repeatability of the bioprocess increased. In contrast to the CDC biofilm reactor and the RAB, this design allows a relatively large sampling number of up to 120 coupons per bioprocess. This leaves more possibilities for designing of experiments e.g. testing cell attachment onto different types of materials (silicone to mimic medical catheters or stainless steel for food industrial purposes) or surface treatments during a single experiment. A futher advantage of the presented system is the ability to follow the development of biofilm formation through sampling and analysis of the biofilms at different time points.

The production of a standardized model biofilm enables the determination of cleaning, removal, and killing efficiency of mechanical procedures and chemical agents (Gattlen et al. 2010). With the possibility to sample the biofilms at any phase of their development, antimicrobial studies can be performed with young, initially mature or mature biofilms. The anitimicrobial action or efficacy of other chemical agents can be determined and their concentration-dosage effect can be adjusted against yeast or specifically agains *R. mucilaginosa*.

The antimicrobial agents have to result in an at least a 4-log reduction of cell number for a fungicidal activity (DIN EN 1275), while > 5 $\log_{10}$ reduction of bacterial CFU has to be found according to DIN EN 1040. Similarly, we would expect that a 4-log reduction of cell numbers is aimed for biofilm removal tests even though no requirements for said tests have been available in international standards so far. With our biofilms up to 10^7 cells per cm^2 could be grown after 6 days p.i. Considering that the detection limit for CFU is about 10 cells per cm^2, the produced biofilm should consist of more than 10^6 yeast cells per cm^2 in order to enable the determination of a removal efficiency up to 5 $\log_{10}$, which is the level usually required for antimicrobial testing against fungi (DIN EN 1275).

3.4 Conclusions

Our aim was to produce a model biofilm with *R. mucilaginosa* for testing the removal efficiency of washing devices (e.g. household washing machines). Therefore, we built biofilms that were stable with sufficient amount of viable cells, protein and polysaccharides. In our study we were able to show that yeast biofilms can be grown in a repeatable manner in a modified bench-top bioreactor after 6 days post inoculation. The

yeast biofilms grown on smooth PP surfaces were similar in terms of amount of organic matter and viable cell number in all five independent reactor experiments. Moreover, it was demonstrated that surface modifications (roughening) increased the surface for attachment but concomitantly also increased the variability of all measured parameters compared to the smooth coupons. We also demonstrated that neither roughness alone nor the surface hydrophilicity is decisive for cell attachment and consequently for biofilm formation for *R. mucilaginosa*. However, for yeast biofilms and their cultivation, more fundamental knowledge still needs to be acquired. In particular yeast cells do not have completely identical biofilm formation behaviour as bacteria. Also cell attachment, cell-cell communication and expression profile during biofilm formation are still vastly unknown. Therefore, the relevance and benefit of yeast biofilms need to be further explored.

Acknowledgements

This work has been financially supported by the Swiss Commission for Technology and Innovation (CTI project 9075.1PFLS-LS). Special thanks go to Max Keller, Joerg Gschwend, and the whole team of Empa's machine shop for the construction of the cylinder for the biofilm reactor. We also thank Kathrin Grieder, Aline Hunziker, Bettina Lanz, Monika Braegger for the microbiological analysis, Barbara Hanselmann for the plasma treatments, Patrick Rupper for XPS analyses, Miele and Treff AG for providing polypropylene material, and Bruno Wampfler for fruitful discussions about the manuscript.

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3.5 Supplementary Data to Chapter 3 (published online)

Analysis of roughness of polypropylene coupons

This supplementary information further details the influence of smooth, rough, and dye-casted (dc) poly(propylene) (PP) coupons (treatments were described in the main text) as substratum on biofilm formation.

Atomic force microscopy (AFM)

The surfaces of the smooth, rough and dcPP were scanned with an Easyscan 2 atomic force microscope (Nanosurf AG, Liestal, Switzerland) using contact mode. The cantilever had a spring constant k = 0.15 N m^{-1} and length of 447 μm.

For the measurement of the surface roughness, an area of 40 μm x 40 μm and 15 μm x 15 μm for the rough coupons was chosen and the following parameters were used. Operating mode: static force: 40 nN, image size: 40 μm size, timeline: 1.5 s/line, 512 points/line. The roughness was determined with Nanosurf Easyscan program. The main parameters were calculated by the software using the equation (1) for the roughness average S_a and (2) for root mean square S_q:

$$S_a = \frac{1}{MN}\sum_{k=0}^{M-1}\sum_{l=0}^{N-1}\left|z(x_k, y_l)\right| \quad (1)$$

$$S_q = \sqrt{\frac{1}{MN}\sum_{k=0}^{M-1}\sum_{l=0}^{N-1}\left(z(x_k, y_l)\right)^2} \quad (2)$$

The surface structure of the dye-casted coupons was more semi-spheres with smoother edges than the smooth coupons (see Figure S3.1 and also Table S3.1). For the rough coupons only a smaller area (15μm x 15 μm) could be analyzed because 40 μm x 40 μm was too large due to structures that were too rough to be analyzed by AFM (Table S3.1).

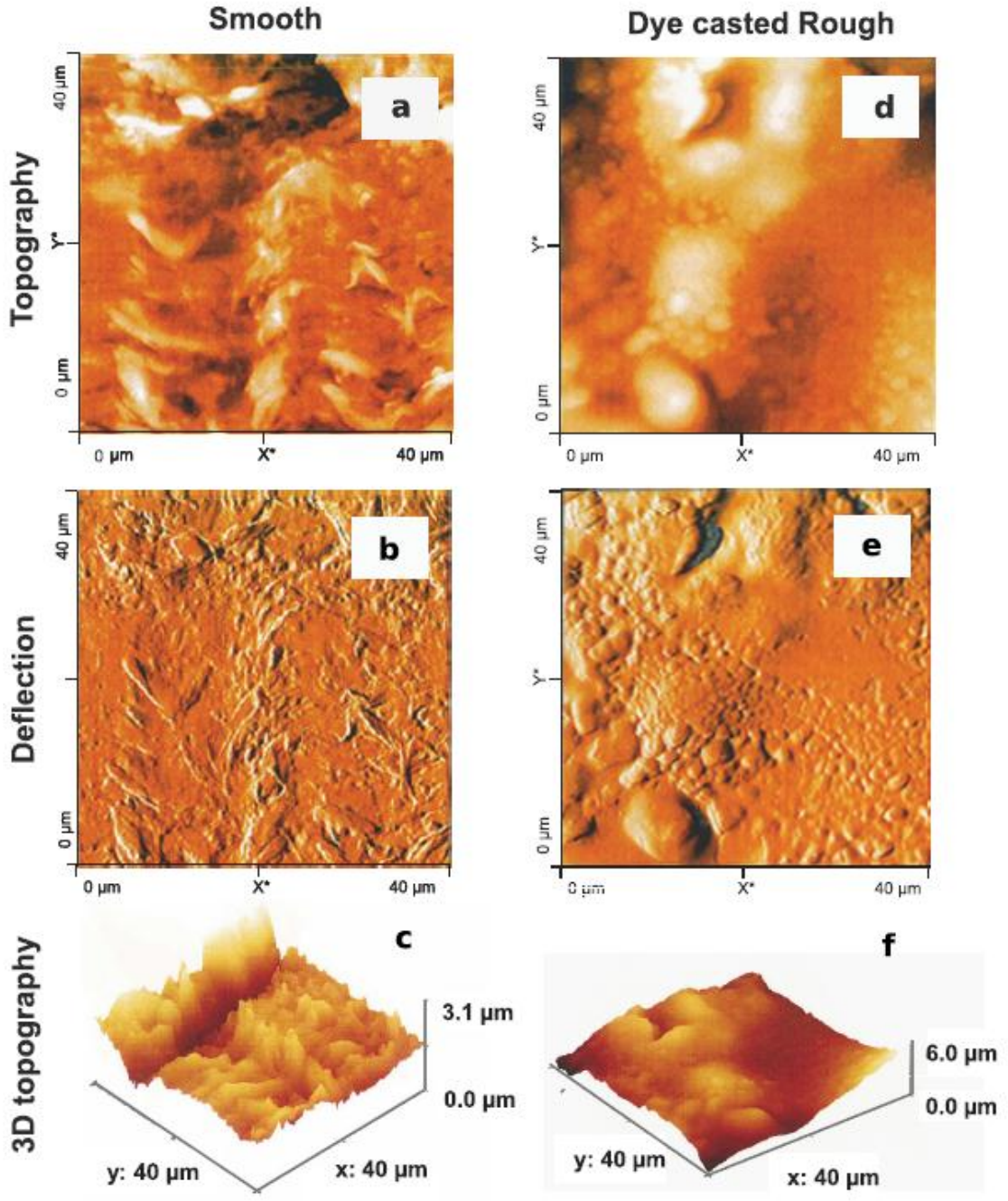


Figure S3.1. AFM micrographs of topography, deflection and 3-dimensional topography of the two different polypropylene surfaces a-c) smooth, d-f) dye-casted rough coupons. For the smooth and dye casted rough surface an area of 40 x 40 μm^2 is represented.

Table S3.1. Results of the AFM analysis for the three tested PP surfaces.

	Overall			Smooth area			Rough area		
	A	**S_a**	**S_q**	**A**	**S_a**	**S_q**	**A**	**S_a**	**S_q**
Smooth	1600 µm^2	179 nm	237 nm	225 µm^2	91 nm	118.5 nm	225 µm^2	150 nm	202 nm
Rough	n. d.	n. d.	n. d.	225 µm^2	73 nm	92 nm	225 µm^2	159 nm	213 nm
Dye-casted	1600 µm^2	428 nm	550 nm	225 µm^2	55 nm	73 nm	225 µm^2	265 nm	372 nm

A: Surface area
S_a: Roughness average
S_q: Root mean square
n. d.: not determined

Repeatability of bioprocess

Statistical analysis

The values of the rough coupons were statistically analyzed in the same manner (normal dis- tribution, box plot, linear regression, two-way ANOVA with and without repetition as it was performed for the smooth coupons (explained in more detail in the main text). In total n = 15 - 18 coupons were used for analysis.

The biofilm was characterized by quantification of the amount of protein and polysaccharides. Viable cell count and optical density was similar from one experiment to another. The overall distribution of the biofilm built on rough coupons from one independent experiment to another experiment was larger than on smooth coupons. Comparison of the group medians (from one experiment to another) revealed relatively large differences. The distribution of the values was relatively large also within a single experiment. The range of variation in experiment No. 3 (see also main text) for protein was more than 500 µg cm^{-2} and for polysaccharide more than 2000 µg cm^{-2}. Also the median from one experiment to another varies relatively strong for protein and polysaccharide. Nevertheless, none of the experiment was completely different because the independent distributions overlapped (Figure S3.2).

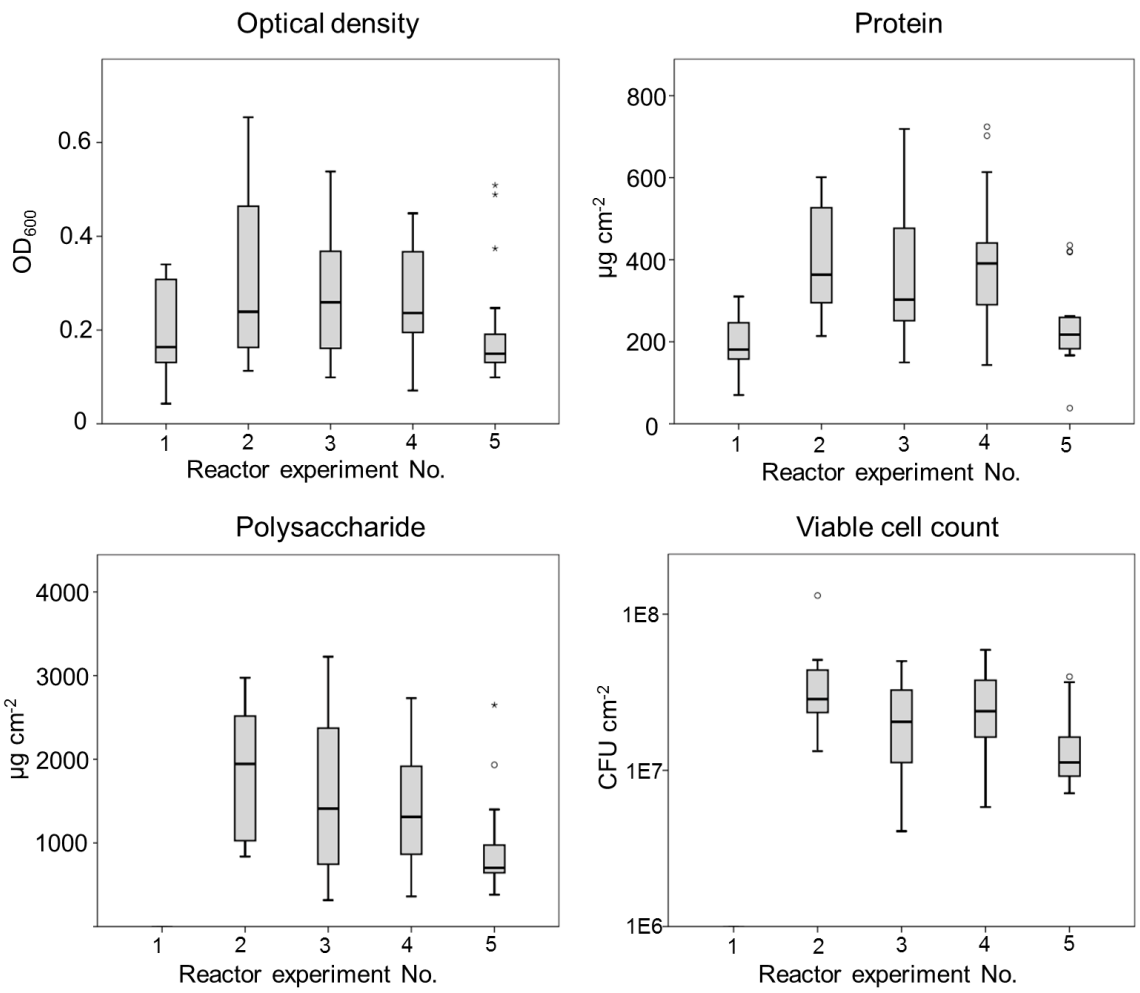


Figure S3.2. Box plot analysis of biofilms formed on rough PP coupons in n = 5 independent reactor experiments. OD_{600}, protein, polysaccharide and viable cell counts were analyzed. Circles: outlier, stars: extreme values.

The vertical alignment of the remaining parameters examined for smooth coupons are shown in Figure S3.3 together with the vertical alignment for protein. Except for protein in experiment No. 2 (see main text), the linear regression was significant for optical density ($p = 0.02$) and viable cell count ($p = 0.008$).

Focusing on the vertical alignment of a single holder with three coupons, it could be observed that gradients existed for all tested parameters (Figure S3.4). However, none of the tested holders showed a significant vertical gradient. This is due to the low number (n = 3) of samples per holder but can also be due to a large heterogeneity between the different samples.

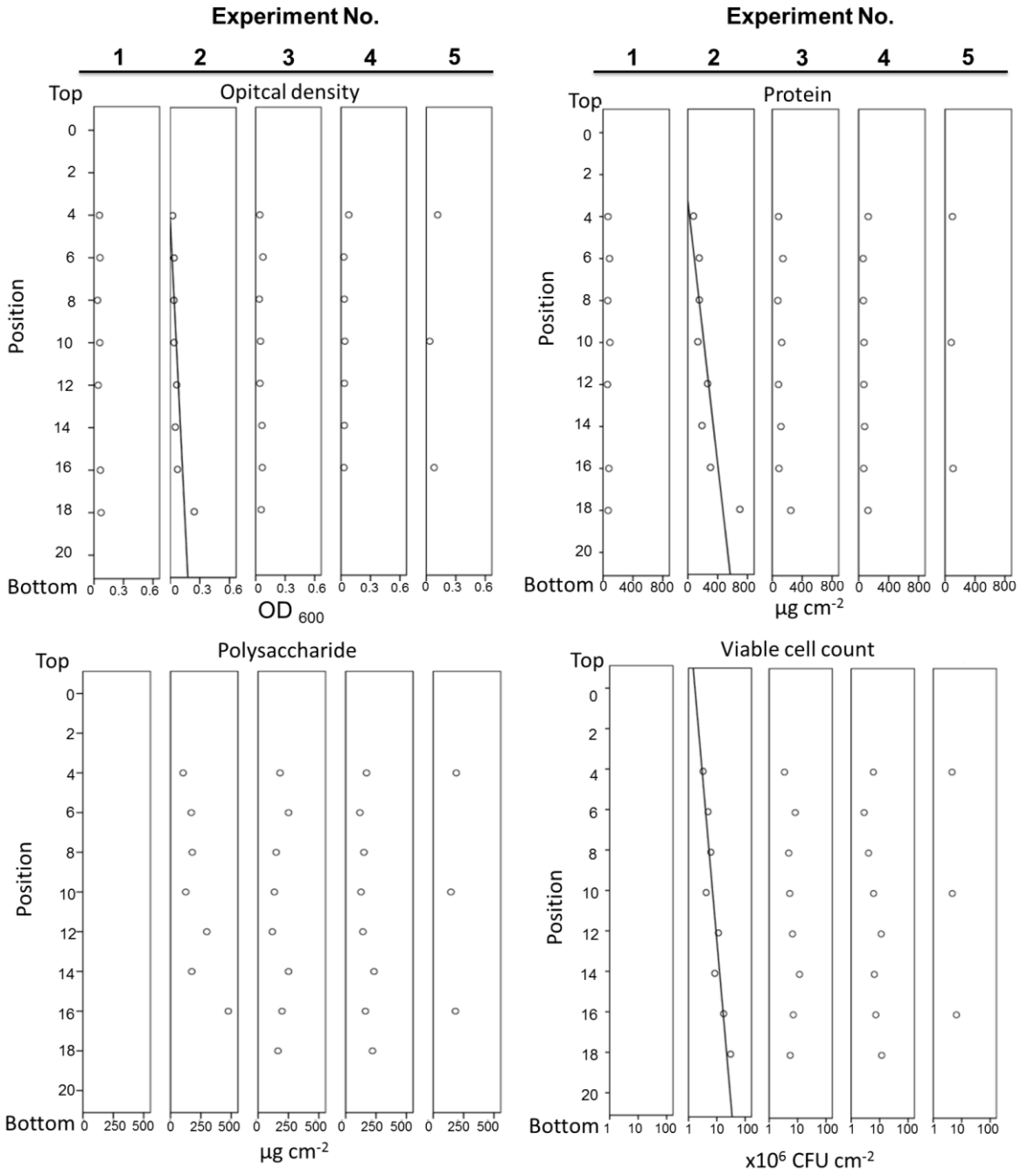


Figure S3.3. Vertical alignment and linear regression analysis from top to bottom of the smooth coupons of each holder analyzed per reactor showing the results for all four tested parameters. $\alpha = 0.05$. The linear regression is only significant for experiment No. 2 for protein, optical density and viable cell count.

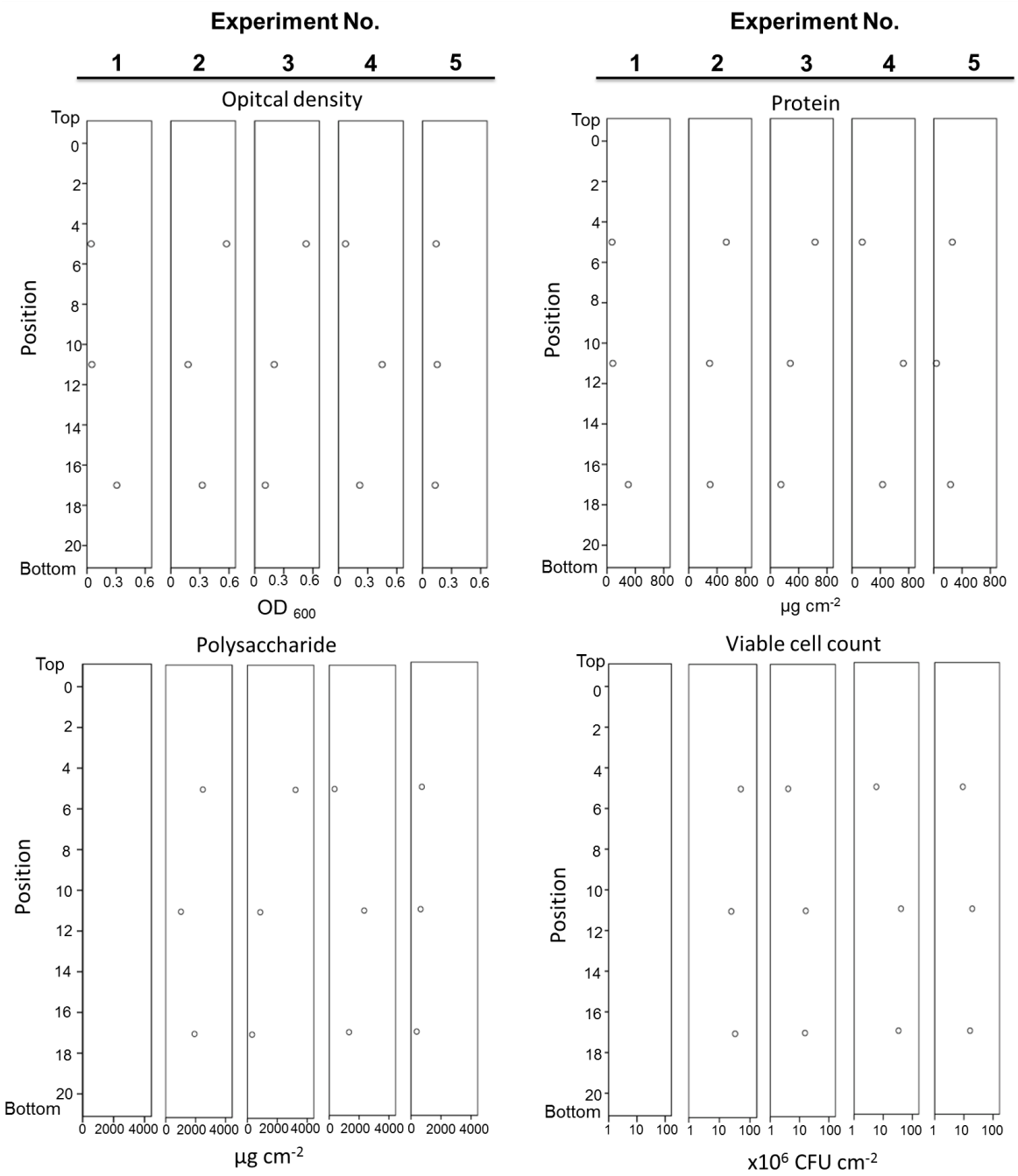


Figure S3.4. Vertical alignment and linear regression analysis from top to bottom of the rough coupons of each holder analyzed per reactor showing the results for all four tested parameters. $\alpha = 0.05$. None of the linear regression analysis was significant.

To determine the source of variability within a reactor experiment, a two-way analysis of variance (ANOVA-2) without repetition was performed. The largest variability within the reactor was observed for comparisons of vertical than for the horizontal positions (exception for OD_{600}) (Table S3.2). The differences from the minimal to maximal mean value between different reactor experiments was 36% for OD_{600}, 52% for protein, 49% for polysaccharide and for viable cell count it was 57%.

Table S3.2. Summary of results for biofilm accumulation at day 6 post inoculation for rough coupons and distribution of the source of variance within a reactor experiment.

Parameter	Components of total variability	Exp. 1	Exp. 2	Exp.3	Exp. 4	Exp. 5
OD_{600}	Average value	0.20	0.32	0.28	0.27	0.20
	Standard deviation	0.10	0.18	0.15	0.11	0.12
Components of total variability	Vertical (%)	38.0	22.3	21.5	26.8	18
	Horizontal (%)	8.2	9.2	32.8	15.3	22.6
	Residual error (%)	53.8	68.5	45.7	57.9	59.5
Proteins	Average value (µg cm^{-2})	194.6	387.7	357.3	407.7	239.8
	Standard deviation (µg cm^{-2})	70.3	131.7	159.6	152.1	99.1
Components of total variability	Vertical (%)	32.1	6.0	12.7	10.1	22.0
	Horizontal (%)	4.2	11.3	29.0	16.1	20.3
	Residual error (%)	63.7	82.8	58.3	73.8	57.7
Polysaccharide	Average value (µg cm^{-2})	n. d.	1809.8	1492.5	1397.6	927.1
	Standard deviation (µg cm^{-2})	n. d.	768.1	855.3	620.6	559.9
Components of total variability	Vertical (%)	n. d.	5.4	11.5	19.1	31.6
	Horizontal (%)	n. d.	13.4	34.5	20.7	19.3
	Residual error (%)	n. d.	81.2	54.0	60.1	49.1
CFU	Average value (CFU cm^{-2})	n. d.	3.6E+07	2.3E+07	2.8E+07	1.6E+07
	Standard deviation (CFU cm^{-2})	n. d.	2.6E+07	1.4E+07	1.5E+07	1.1E+07
Components of total variability	Vertical (%)	n. d.	29.9	23.5	14.4	18.9
	Horizontal (%)	n. d.	4.3	0.4	20.5	9.5
	Residual error (%)	n. d.	65.8	76.1	65.1	71.6

The variance components are shown as percentages of the total variability

n. d.: not determined

4. Analysis of biofilm formation of *Pseudomonas putida* in biofilm minimal biofilm medium and its cultivation in a modified bench-top reactor applying different experimental set-ups for model biofilm production

Abstract

The Gram-negative bacterium *Pseudomonas putida*, a washing machine isolate, was tested for its biofilm formation ability in presence of different concentrations of seven selected elements used in a designed minimal test medium (biofilm minimal medium). The seven elements (C, N, P, Ca, Mn, Fe and Zn) were tested at two different concentrations in 96-well plates. Carbon was the most influential parameter for biofilm formation along with phosphorous (phosphate) and iron.In a next step, *P. putida* was assessed for its biofilm forming ability in a modified 3.7 L bench-top reactor on different test materials (polypropylene, stainless steel, Teflon and PE-1000). Polypropylene was the most appropriate material as substratum and the temporal formation of biofilm was analyzed. The maximal amount of biofilm was built at day 7 post inoculation. Further, it was also determined whether biofilm formation could be accomplished in a repeatable manner in the modified bioreactor smooth polypropylene and on dye-casted polypropylene coupons.

Keywords: nutrients, bioprocess, biofilm formation, polypropylene

My contribution was the cultivation and analysis of the *P. putida* biofilms grown in the reactor with support from Aline Hunziker and Dr. Laurie Mauclaire. Reactor experiments according to experimental set up E was performed by Bettina Lanz and Monika Brägger (ETM).

4.1 Introduction

Biofilm formation is influenced by many different factors such as nutrient availability, substratum for cell adherence, shear forces of the bulk fluid and the type of microorganism. Nutrients present in the surrounding bulk fluid not only provide microbial cell growth and physiological maintenance, but also have an essential influence on the formation and stability of biofilms. Carbon (C), nitrogen (N), phosphorus (P), calcium (Ca^{2+}), iron (Fe^{2+} and Fe^{3+}) and manganese (Mn^{2+}) have already been described in affecting biofilm formation. Single nutrients as well as combinations and ratios between certain elements play a key role in the control and development of biofilms. Several publications deal with the influence of the C:N or C:P ratio with respect to biofilm formation (Rochex and Lebeault 2007). Increased thickness and density are hallmarks of biofilms grown in high nutrient environments (Sutherland 2001). However, it is believed that most of the biofilms are living in oligotrophic environments (http://www.cs.montana.edu).

Besides nutrient availability, substratum material is a further important factor in biofilm formation. Biofilms colonize nearly all thinkable surfaces that are in contact to humid or liquid environments (Sutherland 2001). Therefore, biofilm can be a problem in medical, industrial and private settings. Examples for materials being prone to biofilm colonization are plastics, that are used for wastewater pipes (Mantovi et al. 2003), and stainless that is especially used in food processing (Jullien et al. 2003; Elhariry 2008; Oliveira et al. 2010).

The first goal of this study is to determine the influence of seven nutrients (present in our designed biofilm minimal medium) on the biofilm formation of *P. putida*. Using the experimental set-up based on the „ruggedness test“, a multifactorial method, developed by Youden and Steiner (Youden and Steiner 1975), the influence of seven factors (nutrients) on biofilm formation can be tested. The nutrient assay can easily be performed in 96-well plates, giving insight into the concentration-depedent influence of the tested nutrients. However, untreated 96-well experiments are restricted to young biofilms (24 - 48 h) due to finite nutrient supply and single type of material (usually polystyrene) as substratum.

The second goal is to scale-up the biofilm formation process, using the biofilm minimal medium to grow *P. putida* biofilms in a modified bench-top reactor, assessing i) the experimental set-up, ii) the cultivation period for mature biofilm, iii) biofilm formation on four different test materials (stainless steel, Teflon, PE-1000, PP rough and smooth) as substratum in the reactor and iv) repeatability of the bioprocess. These tests will give

insight into the formation of biofilms for older biofilms (up to 14 days) using a designed medium and modified bench-top reactor for the production of model biofilms.

4.2 Materials and Methods

4.2.1 Cultivation

A single colony of *P. putida* grown on tryptic soy agar (TSA) was taken and transferred into 15 mL of TSB and was grown at 30°C and 150 rpm for 12 h. Ten ml of the overnight culture were harvested and centrifuged (15 min, 4°C, 10'000 x g, Sorvall), the supernatant was discarded and cells were washed in 0.9% NaCl solution and centrifuged again. The supernatant was discarded and the cells were dissolved in different compositions of minimal biofilm medium (Table 4.1).

The inoculum was diluted with fresh medium to achieve an OD (600 nm) of 0.05. This was done with all compositions of minimal medium. Aliquots (200 µL) of this cell suspension were transferred into each well of 3x2 96-well plates (TPP92096, flat bottom, Trasadingen, Switzerland). The well plates were covered and sealed with adhesive tape and cultivated (30°C, 50 rpm 24 h, Lab-Therm LT-W, Kühner AG, Birsfelden, Switzerland). The nutrient limitation experiments were conducted with three colonies (clones) on triplicate 96-well plates. The original biofilm minimal medium was added as a control (8 wells per plate).

4.2.2 Media composition

Two different concentrations of nutrients (designated as high and low concentration) were tested as shown in Table 4.1. The remaining nutrients were constant for all media compositions: 0.25 g L^{-1} $MgSO_4$ x $7H_2O$, 0.2 g L^{-1} Na_2-EDTA. The experimental design and composition of combinations of high and low nutrient concentrations are given in Table 4.2. For a single run two well plates were needed. Experiments No. 1-4 were performed in one well plate and experiments No. 4-8 were performed in the second well plate. All experiments were performed in triplicates.

Table 4.1. Parameters and the values for high (A-G) and low (a-g) nutrient concentrations in the modified biofilm minimal media are shown in comparison to the original biofilm minimal medium.

Parameter: Nutrient	High value ($g\ L^{-1}$)	Low value ($g\ L^{-1}$)	Biofilm minimal medium ($g\ L^{-1}$)
A/a: Glucose (C)	1	0.1	1[a)]
B/b: $(NH_4)_2PO_4$	1.1	0.11	1.1
C/c: KH_2PO_4	0.15	0.015	0.15
D/d: $CaCl_2$x $2H_2O$	0.015	0.00015	0.0015
E/e: $FeSO_4$ x $7H_2O$	0.1	0.001	0.1
F/f: $ZnSO_4$ x $7H_2O$	0.01	0.0001	0.001
G/g: $MnCl_2$ x $2H_2O$	0.0396	0.000396	0.00396

[a]:1 $g\ L^{-1}$ was also used for the screening and biofilm removal experiments in Chapter 2. The original biofilm medium was supplemented with 4 $g\ L^{-1}$ glycerol.

Table 4.2. Experimental design and composition of combinations of high and low nutrient concentrations.

Experiment No.	Factor combinations	Measurement Obtained (Results)
1	A B C D E F G	s
2	A B c D e f g	t
3	A b C d E f g	u
4	A b c d e F G	v
5	a B C d e F g	w
6	a B c d E f G	x
7	a b C D e f G	y
8	a b c D E F g	z

4.2.3 Quantification of biofilms

From the suspension 100 µL were taken and transferred into fresh well plates (Nunc, Denmark). The optical density was measured at 595 nm (Cary Microplate reader, Varian). The remaining suspension was discarded. The biofilms were then washed and quantified with the crystal violet (CV) assay as already described in Chapter 2.2.3.

4.2.4 Growth limitation experiment

4.2.4.1 Statistical analysis

For the statistical analysis of the amount of the planktonic cells and formed biofilms the mean was determined per clone. Per clone the mean of the 3x2 plates (per clone) was taken to determine the effect of each factor (Youden and Steiner 1975). To calculate the absolute differences of the seven parameters (A-a, B-b, C-c, D-d, E-e, F-f, G-g) the equations in Table 4.3 were used.

Table. 4.3. Equations for the calculation of the absolute differences for each parameter of the seven parameters A-G.

Calculation of the absolute differences
$\lvert(\bar{A}-\bar{a})\rvert = \frac{(s+t+u+v)}{4} - \frac{(w+x+y+z)}{4}$
$\lvert(\bar{B}-\bar{b})\rvert = \frac{(s+t+w+x)}{4} - \frac{(u+v+y+z)}{4}$
$\lvert(\bar{C}-\bar{c})\rvert = \frac{(s+u+w+y)}{4} - \frac{(t+v+x+z)}{4}$
$\lvert(\bar{D}-\bar{d})\rvert = \frac{(s+u+w+y)}{4} - \frac{(t+v+x+z)}{4}$
$\lvert(\bar{E}-\bar{e})\rvert = \frac{(s+t+y+z)}{4} - \frac{(u+v+w+x)}{4}$
$\lvert(\bar{F}-\bar{f})\rvert = \frac{(s+u+x+z)}{4} - \frac{(t+v+w+y)}{4}$
$\lvert(\bar{G}-\bar{g})\rvert = \frac{(s+v+w+z)}{4} - \frac{(t+u+x+y)}{4}$

The differences were sorted according to their absolute values. The largest difference indicated the strongest effect upon the method. To test whether the difference was significant, a two-sided t-test was conducted according to equation (1).

$$\frac{|X - x|}{s} \cdot \sqrt{\frac{n}{2}} > t_{N-1,P} \quad (1)$$

s: standard deviation (based on N wells filled with the original biofilm medium, here N = 48 per clone)

N: number of wells with the original biofilm medium (2x3x8=48 wells)

n: number of determinations for X and x, respectively (n = 63-1wells x 4)

P: significance level (95%)

4.2.5 Biofilm reactor

A 3.7 L benchtop biofilm reactor with a rotating cylinder (see Chapter 3) was modified such that the inlet of fresh nutrients was first introduced from the top of the reactor and for later experiments from the middle. In some of the experiments, the fresh medium was aerated in the aeration bottle prior entering the reactor (see details in Table 4.4).

4.2.6 Coupon preparation

Round coupons made of different materials with a radius of 2 mm and a surface of 0.5024 cm^2 were glued with silicon glue (ELASTOSIL® E70-Wacker Chemie AG, Stuttgart, Germany) into the sample holders, whereas trapezoid coupons (surface: 0.49 cm^2) were directly inserted into the holders (see Chapter 3). All materials (see Table 4.4) were placed in ethanol 70% and sonified for ca. 10 min. The ethanol was replaced with fresh technical grade ethanol and the coupons were air-dried afterwards. The holders with the mounted coupons were autoclaved prior to mounting them on the non-sterile cylinder in the reactor.

4.2.7 Experimental set-up

In total five individual experimental set-ups were tested (A-E). Experiment A comprised four independent reactor experiments always applying the same test conditions. Experiment B, testing four different types of materials, was performed once. In experiment C smooth PP was used in a single reactor experiment to assess the kinetics of biofilm

formation. The experimental conditions for experiment D and E were identical (cultivation period, location of medium inlet). In experiment D smooth PP as well as dcPP were used and in E only dcPP. In addition, Experiment E was conducted by other lab-workers running five independent reactor experiments. A summary of the different test conditions is given in Table 4.4.

Table 4.4. Materials and their biofouling test conditions.

Experimental set-up (Bioprocess)	Number of independent reactor experiment	Cultivation period (days p.i.[a])	Materials	Holder	Medium inlet
A	4 (No. 1-4)	13	PP round (Miele)	Holder a-f	Top, BF medium via bottle
B	1	13	PE-1000, smooth, round	Holder a-c	Top, BF medium directly into reactor
			Stainless steel (rough, trapezoid)	Holder d	
			PP round (Miele)	Holder e	
			Teflon (smooth trapezoid	Holder f	
C	1	1, 3, 7, 10, 14, 21	PP round (Miele)	Holder a-f	Middle, medium directly into reactor
D	1	6	Dye-casted PP trapezoid (Treff)	Holder a-c	Middle, medium directly into reactor
			PP trapezoid (Miele)	Holder d-f	
E	5 (No. 1-5)	6	Dye-casted PP trapezoid (Treff)	Different holders	Middle, medium directly into reactor

[a] post inoculation

4.2.8 Cultivation of pre-inoculum and inoculum

Pseudomonas putida, a washing machine isolate (see Chapter 2), was used as test organism. A pipette tip-full (ca. 10 µl) of a *P. putida* cryo culture was inoculated into 5 mL TSB/nutrient broth (5 g L^{-1} peptone, 3 g L^{-1} meat extract, pH = 7.0) supplemented with 4 g L^{-1} glycerol, 30°C, 150 rpm) overnight and streaked on nutrient/TS agar plates with 4 g L^{-1} glycerol and incubated at 30°C.

One colony was picked and inoculated into 10 mL nutrient broth with glycerol and cultivated at 30°C, 150 rpm for ca. 12 h. The suspension (7.5 mL) was then transferred into a baffled shake flask with 150 mL of fresh nutrient broth supplemented with glycerol as C-source and cultivated at 30°C for 9 - 12 h. Optical density (OD) measurement at 600 nm were done at different time points in order to check the growth phase of the cells. Cells in the late exponential growth phase (OD_{600} of 2.5 -3.5) were inoculated into the reactor.

4.2.9 Set up of reactor and sterilization

The biofilm reactor with the mounted test coupons was filled with 2.5 L of 30% nutrient broth, supplemented with 4 g L^{-1} glycerol, and heat sterilized (30 min at 121°C). Overpressure (1 bar) was kept during the cooling process by introducing pressurized air via a 0.22 µm filter (Millipore) to avoid contamination during inoculation of the reactor.
The external aeration bottle filled with 500 mL of the same growth medium, as well as the peripheric system were autoclaved separately. The external aeration loop was aseptically connected to the reactor and sterile-filtered pressurized air was added to the aeration bottle. The aerated cultivation medium was pumped into the reactor due to the overpressure in the bottle. The medium in the reactor was constantly pumped into the aeration bottle via a peristaltic pump (Periplex, Bioengineering, Wald, Switzerland). The aerated fresh medium was introduced into the reactor at the surface culture broth in experiment A and B, and from the middle of the reactor for experiments C-E as countermeasure to limit the vertical gradient. In A the fresh biofilm medium was introduced to the aeration bottle and later transferred into the reactor, whereas in experiment B-E the biofilm medium went directly into the reactor by circumventing the aeration bottle, nutrients primarly supply the cells within the reactor are not consumed first by the cells in the aeration bottle.

4.2.10 Inoculation and bioprocess - Cell cultivation and reactor inoculation

The cells in the late exponential phase were inoculated into the reactor at 30°C and 120 rpm. Cells in suspension were collected ca. every hour ± 30 min and OD_{600} was monitored. When the maximal growth rate μ_{max} (0.4 h^{-1}) was reached, the washout of non-adhered cells was initiated by switching to continuous cultivation using biofilm minimal medium containing glycerol. The initial dilution rate was set to 0.5 h^{-1} (time course experiment) and ca. 0.8 -0.9 h^{-1} (remaining reactor experiment), respectively. This wash-

out phase lasted for ca 13 ± 1 h and was followed by regular growth conditions with a dilution rate of 0.12 h^{-1} for further cultivation.

4.2.11 Harvesting of biofilm coupons

The coupons containing biofilms were harvested after 13 days post inoculation (p.i.) (experiment A and B) or after 7 days p.i. (D and E). In experiment C coupons were harvested at six different time points during a three-weeks period. One holder with 6 coupons was harvested at day 1, 3, 7, 10, 15, and 21 p.i. The samples were taken aseptically through an opening in the lid in a low particle environment.

4.2.12 Quantification of initially mature biofilms

After taking each holder out of the reactor, the coupons were removed from the holder and dipped into 0.9% NaCl solution to remove loosely attached cells and finally transferred into 5 mL of sterile 0.9% NaCl where they were sonified (Branson sonifier tip, 10% amplitude, 30 s, 1 s pulse on, 1 s pulse off) at 0°C. Cell suspension was vortexed and 1 mL was taken for OD measurement at 600 nm, 0.5 mL for protein (micro BCA kit, Invitrogen, manufacturer instruction/manual), 2 mL for polysaccharide quantification based on the Dubois assay and 0.5 mL for viable cell counts (10-fold dilution series).
OD measurements, protein and polysaccharide quantification and viable cell counts were conducted as described in Chapter 3.

Polysaccharide quantification. A standard curve D(+)-glucose (0 - 35 µg mL^{-1}) was prepared prior to sample quantification. Two mL of sample were taken and 50 µL of 80% (w v^{-1}) phenol dissolved in water was added and the sample was vortexed. Five mL 98% sulphuric acid was added within 20 - 30 s in the middle of the reagent glass to ensure best mixing and vortexed for 1 min and cooled down for 10 min. Then, the samples were transferred into a water bath and incubated at 26°C ± 1°C for 20 min. The optical density of the samples was measured at 485 - 490 nm (Dubois et al. 1956). When the samples exceeded the values of the standard curve, samples had to be diluted with 0.9% NaCl before adding phenol and sulphuric acid.

4.2.13 Statistical analysis

Average and standard deviation were calculated. Box plot, one-way ANOVA and regression analyses were conducted with SPSS (version 19, IBM) and excel (Microsoft), respectively. In experimental set up B (4.3.4) the data from holder a, b and c were averaged prior to run a one-way ANOVA to compare it with the holder d-f.

4.3 Results and Discussion

4.3.1 Influence of nutrients upon biofilm formation

The sequence of the influence of the nutrient on each of the three clones is given in Table 4.5. The results on planktonic cells indicated that the phosphate concentration is the most influencial factor, followed by Fe (clone 1 and clone 2). For clone 3 the most influencial factor upon planktonic cells was N followed by P.

For biofilm formation limitations C had the largest impact on all three clones. Limitations of P and Fe also affected biofilm formation with a lower impact than C. Interestingly, high Zn concentration rather led to smaller amount of biofilm formation. Mn and Ca had a subordinat effect upon biofilm formation. Similar to Zn, higher Mn concentration led to lower amount of biofilm.

Little differences between the three clones could be observed for cell suspension and the biofilm formation. For biofilm formation the effect of C and Zn was the same for all clones. The effects of the other nutrients were more variable between the clones. In terms of biofilm formation clone 1 and clone 2 behaved more similarly than clone 3. More differences between the clones were observed for influence of the nutrient upon cell suspension

It was also tested whether these effects are signifcant. In only few cases the differences in biofilm formation or on cell suspension was not significant. All of the tested nutrient had a significant influence on the ratio of cell attachment (CV/OD) (Table 4.6).

According to the results, the planktonic growth and the biofilm formation can be better controlled by limiting the content in C, P and Fe.

Table 4.5. The order of influence of each tested element on the three *P. putida* clones.

Order of Influence	Influence of the nutrient on planktonic cells		
	Clone 1	**Clone 2**	**Clone 3**
1	KH_2PO_4	KH_2PO_4	$(NH_4)_2PO_4$
2	$FeSO_4$ x $7H_2O$	$FeSO_4$ x $7H_2O$	KH_2PO_4
3	$(NH_4)_2PO_4$	Glucose (C)	$FeSO_4$ x $7H_2O$
4	Glucose (C)	$(NH_4)_2PO_4$	$MnCl_2$ x $2H_2O$
5	$ZnSO_4$ x $7H_2O$	$CaCl_2$x $2H_2O$	Glucose (C)
6	$CaCl_2$x $2H_2O$	$MnCl_2$ x $2H_2O$	$ZnSO_4$ x $7H_2O$
7	$MnCl_2$ x $2H_2O$	$ZnSO_4$ x $7H_2O$	$CaCl_2$x $2H_2O$

Order of Influence	Influence of the nutrient on biofilm formation		
	Clone 1	**Clone 2**	**Clone 3**
1	Glucose (C)	Glucose (C)	Glucose (C)
2	KH_2PO_4	KH_2PO_4	$FeSO_4$ x $7H_2O$
3	$(NH_4)_2PO_4$	$FeSO_4$ x $7H_2O$	KH_2PO_4
4	$FeSO_4$ x $7H_2O$	$(NH_4)_2PO_4$	$CaCl_2$x $2H_2O$
5	$CaCl_2$x $2H_2O$	$MnCl_2$ x $2H_2O$	$(NH_4)_2PO_4$
6	$MnCl_2$ x $2H_2O$	$CaCl_2$x $2H_2O$	$MnCl_2$ x $2H_2O$
7	$ZnSO_4$ x $7H_2O$	$ZnSO_4$ x $7H_2O$	$ZnSO_4$ x $7H_2O$

Table 4.6. The influence of the chosen nutrients on planktonic cells, biofilm formation and the ratio between planktonic cells and biofilm formation.

Nutrient	Influence on planktonic cells	Influence on biofilm formation	Influence on ratio between biofilm and planktonic cells
Clone 1			
Glucose (C)	Significant	Significant	Significant
$(NH_4)_2PO_4$	Significant	Significant	Significant
KH_2PO_4	Significant	Significant	Significant
$CaCl_2x\ 2H_2O$	Significant	Significant	Significant
$FeSO_4$ x $7H_2O$	Significant	Significant	Significant
ZnSO4 x $7H_2O$	Significant	Significant	Significant
$MnCl_2$ x $2H_2O$	Significant	Significant	Significant
Clone 2			
Glucose (C)	Significant	Significant	Significant
$(NH_4)_2PO_4$	Significant	Significant	Significant
KH_2PO_4	Significant	Significant	Significant
$CaCl_2x\ 2H_2O$	Significant	Significant	Significant
$FeSO_4$ x $7H_2O$	Significant	Significant	Significant
ZnSO4 x $7H_2O$	Significant	Not significant	Significant
$MnCl_2$ x $2H_2O$	Significant	Significant	Significant
Clone 3			
Glucose (C)	Significant	Significant	Significant
$(NH_4)_2PO_4$	Significant	Not significant	Significant
KH_2PO_4	Significant	Significant	Significant
$CaCl_2x\ 2H_2O$	Not significant	Significant	Significant
$FeSO_4$ x $7H_2O$	Significant	Significant	Significant
ZnSO4 x $7H_2O$	Significant	Not significant	Significant
$MnCl_2$ x $2H_2O$	Significant	Not significant	Significant

Significance level: 0.05

Based on reports in literature, the macroelements carbon, phosphorous and nitrogen play an important role in biofilm formation. Carbon is the main part of the backbone of nearly all polymeric substances and also part of EPS of biofilms, while P is part of nucleic acids and cell wall components. Nitrogen is needed for the synthesis of amino acids (proteins) and other essential components (e.g. nucleotides).

Low nutrient environments especially with P-limitation, are believed to trigger biofilm formation. For instance, biofilms of *Agrobacterium tumefaciens* exposed to phosphorous limitation in flow cells resulted in increased biomass and surface coverage compared to static conditions, while the thickness under both conditions remained unchanged (Danhorn et al. 2004). Fang and co-workers (Fang et al. 2008) observed that biofilm forming cells, grown under low phosphorus concentrations, enhanced cell growth after addition of phosphorous but also decreasing the amount of produced extracellular polymeric substances (EPS) up to 81% (w w^{-1}). Thompson and co-workers (2006) have found that the C : N : P ratio plays an important role in biofilm formation in *Enterobacter claocae* and *Citrobacter freundii*. In their study, the ratio of C : N : P of 334 : 38: 5.6 resulted in an increased number of attached cells.

The effect of a low iron environment on biofilm depends on the microbial strain. In motile strains, such as *Pseudomonas aeruginosa, P. fluorescens* or *Vibrio cholerae,* low iron environments reduce biofilm development. However, it was demonstrated that high iron concentration (100 µM) triggered cell aggregation and the formation of biofilms, while hundred times lower concentrations enhanced motility and formation of planktonic cells (Berlutti et al. 2005). Calcium is also functioning as co-factor for polysaccharide synthesis (Huang et al. 1994). To a certain extent, it stabilizes the biofilm structure similar to Mg^{2+}. However, excess of calcium can also trigger the detachment of cells (Huang 1994). The influence of zinc is not reported in terms of bacterial biofilm formation. Manganese also belongs to ions that serve as *co*-factors in superoxid and plays a major role in the detoxification in most bacteria (Arirachakaran et al. 2007).

In several studies, it has been shown that nutrient concentrations have an inducing effect on the cells to transform from planktonic to attached (biofilm-forming) state (O'Toole et al. 2000) and on the morphology/morphotype of biofilms (Srinandan et al. 2010). Interestingly, it had been reported that the concentration of some nutrients can have an effect on biofilm formation mainly at the beginning and less during the biofilm development (Kim and Frank 1994).

Taking all this together, controlling nutrient levels may help to enhance the transition form planktonic to attached cells as well as reduction of biofilm formation in industrial systems, e.g. recirculating cooling water system.

4.3.2 Reactor experiment with *P. putida*

4.3.2.1 Four independent reactor experiments conducted with experimental set-up A: Feasibility study for repeatable biofilm formation

After determining the influence upon biofilm formation (P-limitation), *P. putida* biofilms were grown in biofilm reactors to achieve production of large series of thick biofilms.

Although all experiments were conducted under the same experimental conditions, a large variability between the four independent experiments was observed. In particular, the results of experiment No. 1 and No. 2 have a higher similarity (median and range of values) than the latter two experiments independent of the tested parameters.

Reactor experiment No. 3 and 4 showed a tendency for lower values (OD_{600} and polysaccharide). However, for the viable cell count, experiment No. 3, showed the highest median and experiment No. 4 had the largest range of variation. It is probable, that the large variations between experiments occurred randomly (e.g. sampling errors or colony fitness). It has been observed that during sampling, i.e. stopping the rotating cylinder or the removal of holders from the reactor, often caused loss of biofilm due to unstable biofilm architecture. Stopping of the rotating cylinder was rather harsh event because the liquid was still rotating causing further shear upon the biofilms. This could explain the low polysaccharide values in reactor experiment No. 3 and 4 when losely attached EPS detached from the biofilm. Fitness of the cells is also an important factor. Because the inoculum for the bioprocess was based on a single colony, the chosen colonies could have been less fit than during the first two experiments (No. 1 and 2) because during batch cultivation the cells required more time to reach the maximal growth rate. Due to the selection, it is also possible that a colony was chosen that tends to produce more protein than polysaccharides, as seen in experiment No. 4. (protein quantification for experiment No. 3 was not conducted). The amount of viable cell counts were similar for experiments

No. 1, 2 and 4. The median of viable cells was between 10^8 to 10^9 cells cm^{-2}. In experiment No. 3 the median was 0.5-1-log higher.

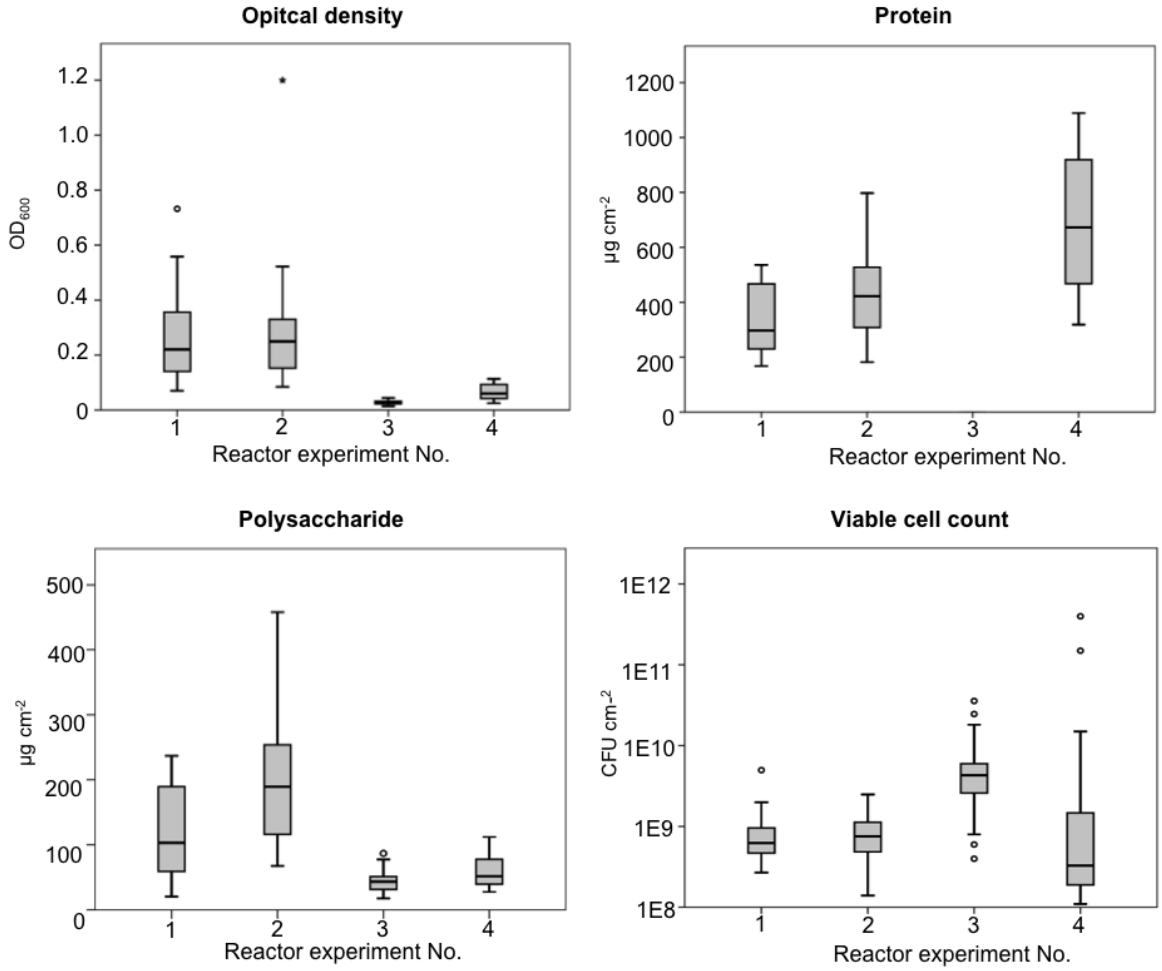


Figure 4.1. Box plots of four independent reactor experiments for optical density, amounts of protein and polysaccharide and viable cell count performed with experimental set-up A. The protein amount in reactor experiment No. 3 was not determined.

To determine the vertical gradient, a regression analysis was conducted (Figure 4.2). In experiment No. 2 a negative vertical gradient for *P. putida* biofilms for all parameters could be observed indicating higher biofilm amounts on the top part of the reactor rather than towards the bottom.

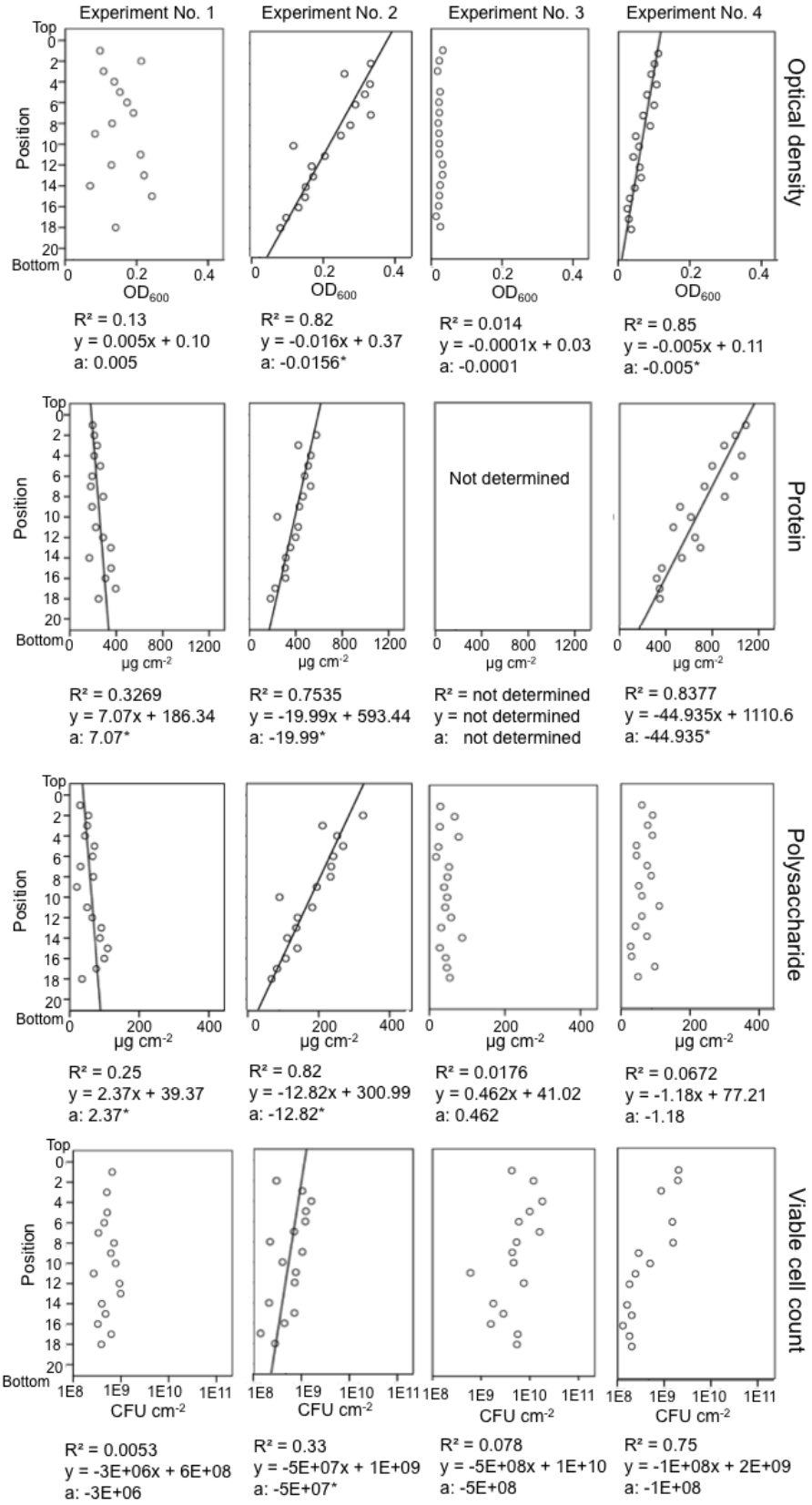


Figure 4.2. Holder a was analyzed for vertical gradient for all tested parameters for the four independent experiments conducted with the experimental set-up A. Regression analysis was significant (*) with $\alpha = 0.05$

Insufficiency of available nutrients was first assumed because the fresh medium first encountered the cells in the aeration bottle before being pumped into the reactor. The cells in the bottle might consume nutrients so that not sufficient nutrients are left for the cells within the reactor. In addition, it is very likely that the position of the medium inlet is of greater importance for spatial gradients and consequently for repeatable biofilm formation. In all these experimental set-ups the medium inlet into the reator was at the surface of culture broth. Mixing experiment revealed that when fresh nutrients entered the reactor at the surface of the culture broth, it required up to 2 min to be completely mixed in the reactor (data not shown). These findings lead to the assumption that long mixing times are responsible for the spatial gradients.

In summary, the repeatability of the bioprocess was not satisfactory under the tested cultivation conditions and reactor set-up. Large spatial variability between samples (within a single reactor experiment) is mainly a result of the the vertical gradient.

4.3.2.2 Single reactor experiments with experimental set-up B: Fouling properties of different materials

In this experiment only one reactor experiment was conducted to test if other materials are more prone to biofouling than the smooth PP, which is used in commercial washing machines and served here as reference material. The biofilms of *P. putida* formed on roughened stainless steel, Teflon and on two types of plastics (polypropylene PP and polyethylene PE-1000) were harvested and quantified 13 days after inoculation as previously described.

PE-1000 showed relatively good fouling properties but the material itself was rather soft and prone to scratches (which occured through simple handling when the coupons were mounted onto the holders). Also roughened stainless steel, a material usually known to enable biofilm formation, did not show any significantly increased biofilm formation. Teflon was used as a negative control due to its ability to decrease fouling because of its low wettability (Demling et al. 2010). However, it was as also prone to biofilm formation, like all the other tested materials (Figure 4.3).

Focusing on each single holder, holder c significantly differed from the other two holders (a and b) in terms of OD_{600} and protein, which could be referred as sampling errors. For the polysaccharide amount, holder c also deviated regarding the medians and range of variance of the datasets in comparison to the other holders (a, b, d-f) (Figure 4.3).

The alignment of the biofilm from top to bottom was checked for a vertical gradient. For OD_{600} and protein a significant linear gradient could be observed in case of holder a (PE-1000), holder d (stainless steel), holder e (PP) and for holder f (Teflon). Neither for the polysaccharide nor for CFU data a linear relationship could be determined.

None of the tested material showed a significantly better fouling property in terms of CFU (ANOVA, $p = 0.174$).

For further experiments smooth PP was still the best option as it was not prone to scratches unlike PE-1000. Further, the production of PP coupons is much cheaper and easier to cut than stainless steel and offer better constrast for staining.

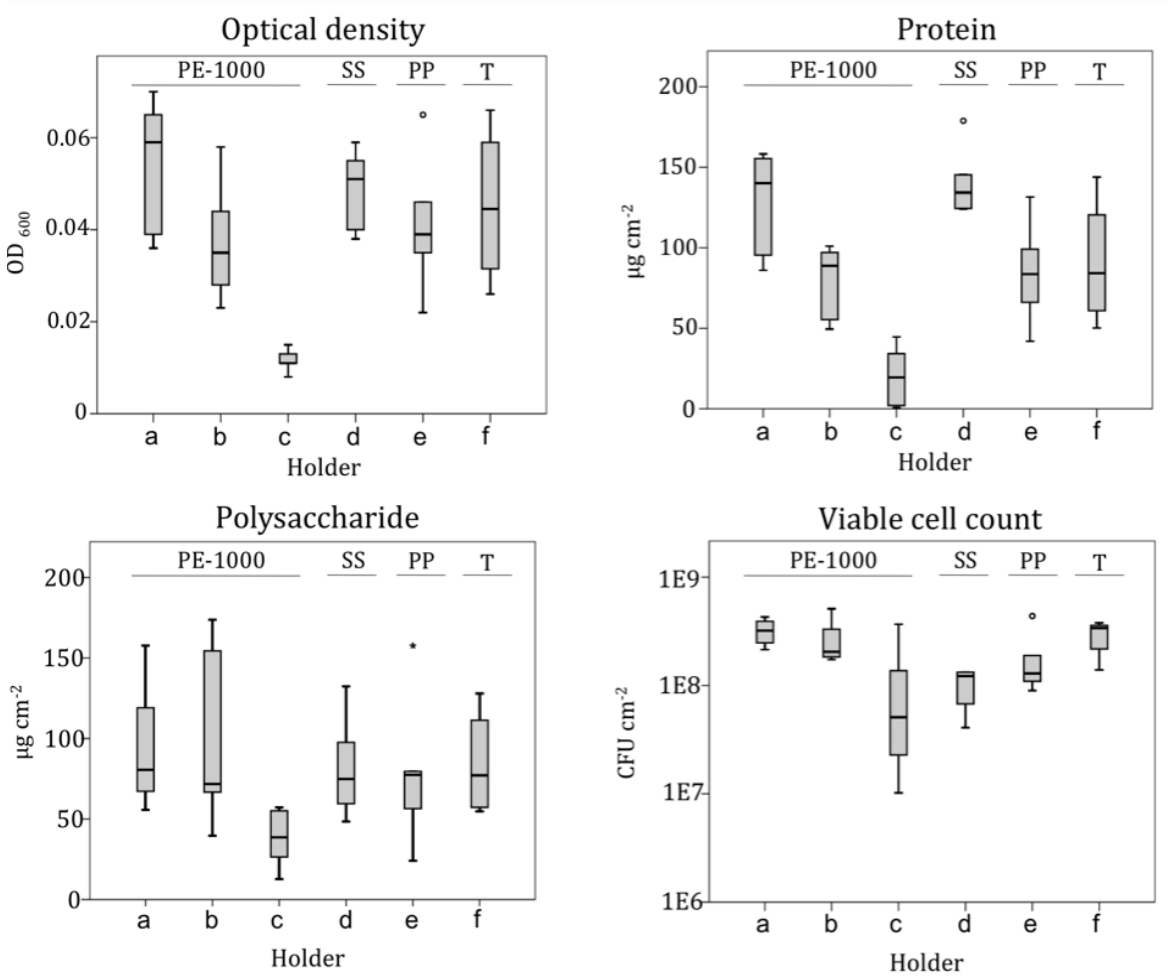
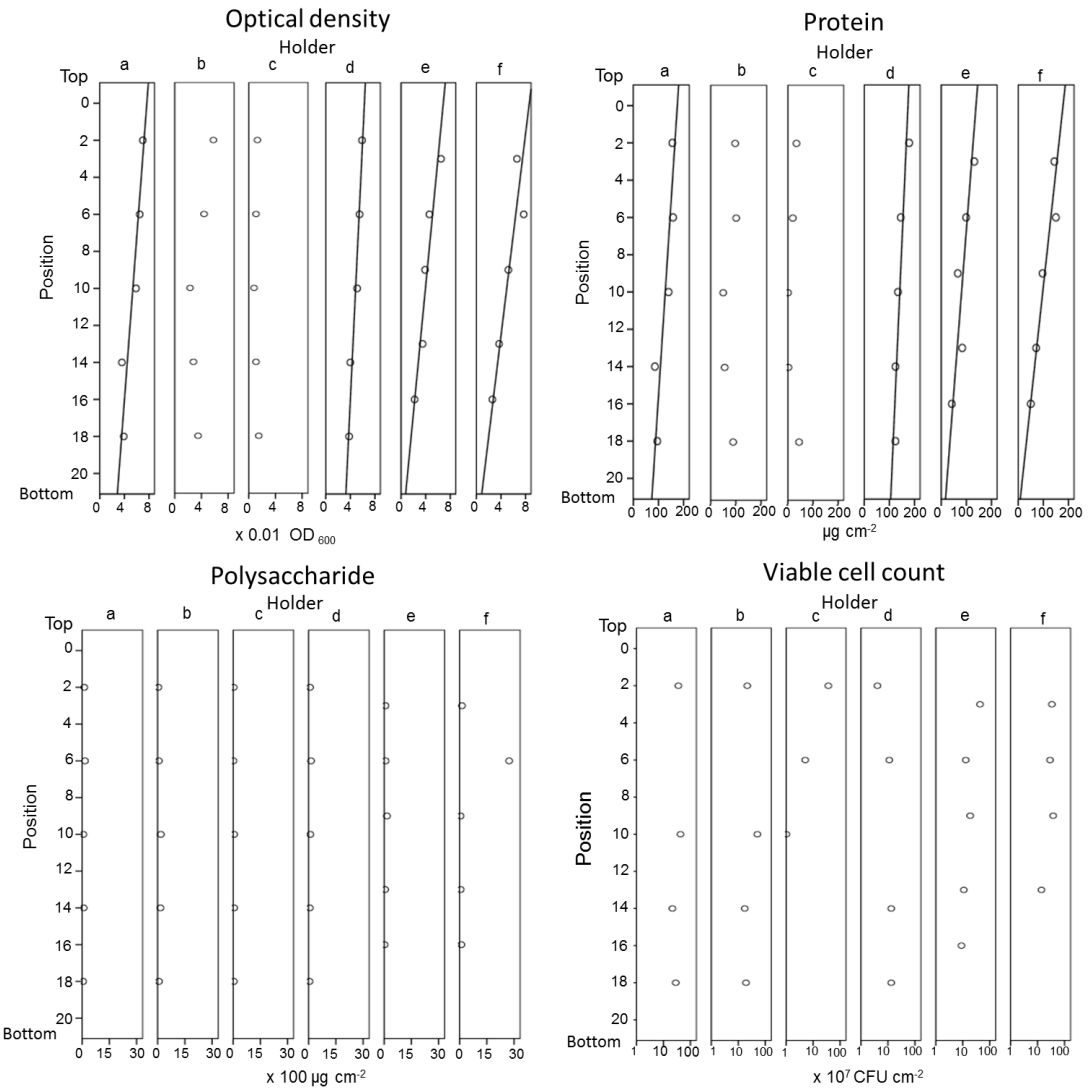


Figure 4.3. Box plot of the tested materials. PE-1000 (holder a-c), stainless steel (holder d), PP (holder e) and Teflon (holder f) were tested for their fouling properties. Bold line: median, circle (°): outlier. Whiskers: minimal and maximal values.



OD_{600}	Holder a	Holder b	Holder c	Holder d	Holder e	Holder f
R^2	0.87*	0.5	0.059	0.97*	0.92*	0.85*
y	-0.0023x+0.077	-0.0016x+0.053	0.0001x+0.011	-0.0017x+0.065	-0.0029x+0.069	-0.0037x+0.086
a	-0.0023	-0.0016	0.0001	-0.0017	-0.0029	-0.0037
Protein						
R^2	0.8*	0.16	0.0007	0.81*	0.79*	0.92*
y	-4.81x+175.13	-1.55x+93.88	-0.081x+19.40	-3.89x+178.01	-5.749x+138.51	-8.07x+178.38
a	-4.81	-1.55	-0.081	-3.89	-5.749	-8.07
Polysaccharide						
R^2	0.59	0.17	0.31	0.39	0.21	0.14
y	-5.10x147.08	3.79x+63.323	1.67x+21.35	-3.978x+119.99	-4.271x+119.2	-84.968x+1411.8
a	-5.1	3.79	1.67	-3.98	-4.27	-84.97
CFU						
R^2	0.28	0.03	0.83	0.72	0.4	0.35
y	-7E+06x 4E+08	-4E+06x+E3+08	-4E+07x + 4E+08	-6E+06x+5E+07	-6E+06x+2E+08	1E+07x + 4E+08
a	-7E+06	-4E+06	-4E+07	-6E+6	-6E+06	1.E+07

Figure 4.4. Vertical alignment of protein of the 6 holders. a–c: PE-1000, d: stainless steel, e: PP, f: Teflon). Regression analysis was significant (*) with $\alpha = 0.05$, R^2: coefficient of determination of a linear regression, y: equation of the linear regression model, a: coefficient of the slope.

4.3.2.3 Single reactor experiment with experimental set-up C: Temporal development of biofilm formation

This experiment was dedicated on examining the temporal development of *P. putida* biofilms grown over a period of three weeks, in order to determine maximal biomass production (CFU, proteins and polysaccharides) for subsequent cleaning tests.

On day 1 p.i. the viable cell counts were very low (7 x 10^3 CFU cm^{-2}). This could be because only a low number of cells was able to attach or because they were adjusted to growth on minimal medium and had difficulties to be cultivated on tryptic soy agar (TSA). On day 3 p.i., *P. putida* produced 1.97 x 10^7 CFU cm^{-2}. During the cultivation period the CFU started to increase until day 7 p.i. to 1.64 x 10^8 CFU cm^{-2}. The amount of CFU cm^{-2} remained stable for the following three days and started to decrease constantly (Figure 4.5a). The protein amount was low until day 3 p.i., increased from thereon to day 7 p.i., and did not significantly change until the termination of the experiment (Figure 4.5b). The amount of polysaccharides remained relatively stable over the whole three weeks of cultivation (Figure 4.5c). The increase of polysaccharides at day 7 p.i. to 116 µg cm^{-2} was not significant.

The reason for the low amount of protein at day 3 p.i. could be due to an analytical error because the other two parameters were comparable to the results of the consecutive sampling days. It is less likely that the cells needed several days to express the system for protein production.

It was concluded that the most appropriate cultivation period for biofilm formation of *P. putida* is 7 days p.i.. Sufficient biofilm (polysaccharide, protein and viable cells) was produced for the main application, namely the efficiency tests of washing detergents. After day 7 p.i., the maximal biomass has been reached and further cultivation did not increase the overall biomass. While the amount of polysaccharides and proteins reached a plateau, the number of viable cells started to decrease.

Based on these results the follow-up experiments were all harvested at day 7 p.i. This finding is of great relevance because shorter cultivation periods will also lower the costs for maintenance of the system (personnel, electricity, medium etc.).

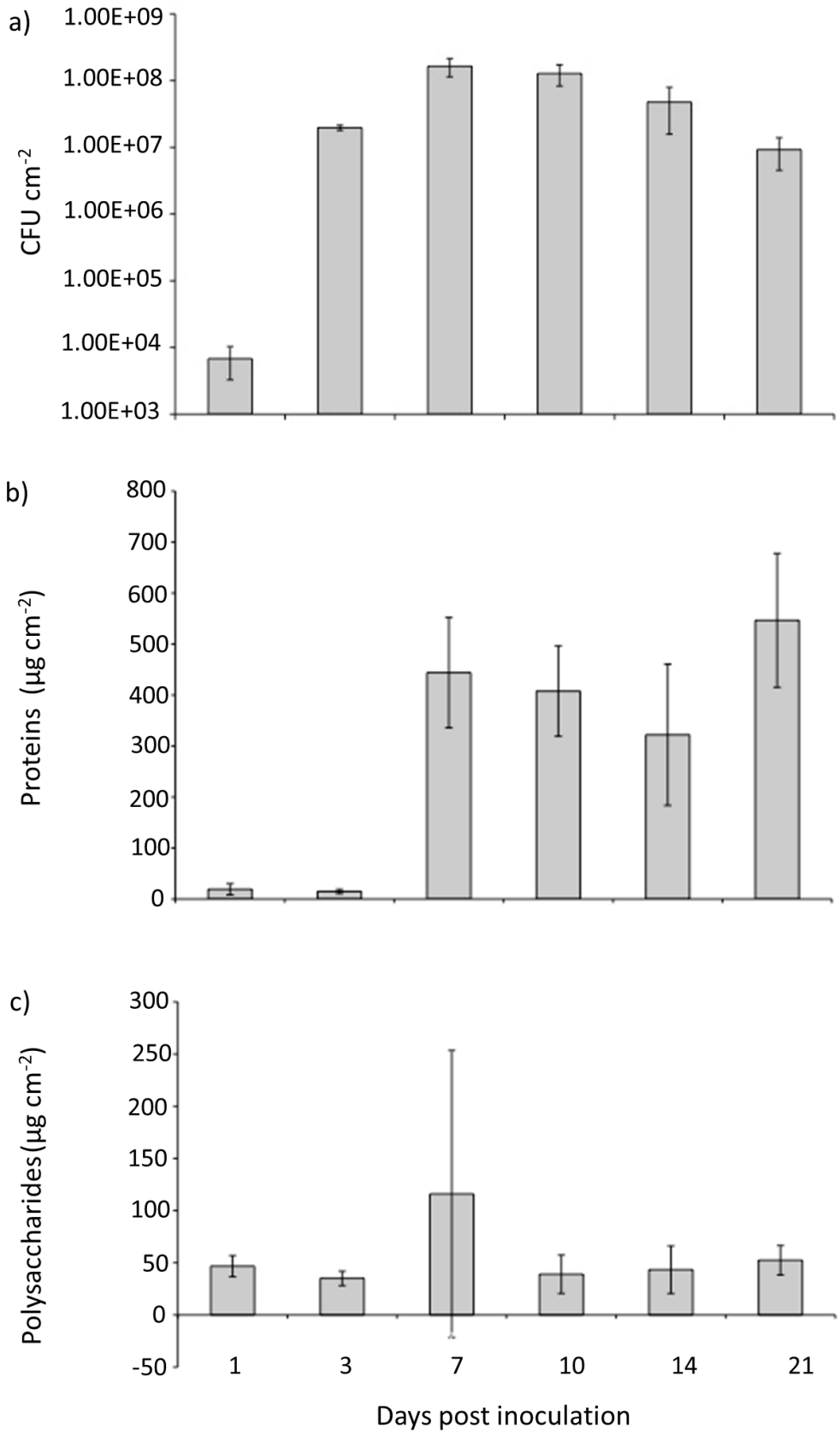


Figure 4.5. Time course of biofilm development of *P. putida* over 21 days post inoculation. Mean values and standard deviation of a) the colony forming units (CFU) per cm^2, b) amount of proteins per cm^2, and c) amount of polysaccharides per cm^2, (n=6).

4.3.2.4 Reactor experiment with experimental set-up D: Comparison of smooth and dye-casted PP

Dye-casted polypropylene coupons were formed via injection molding. They have the advantage, that they did not have to be processed further (cutting and roughening). This saved a lot of time and would increase the repeatability of the coupon production. Therefore, it was also tested if the fouling property was similar to smooth PP used as washing machines material.

The surface structure of both types of coupons was different (see Figure S3.1 supplementary informations of Chapter 3). We expected that rough surfaces tend to promote colonization, but as already observed with yeast (Chapater 3) the dcPP are less affected than smooth PP.

In our experiments, it was observed that the fouling property of the dye-casted (dc) roughened coupons is in general lower than for the cut, smooth PP coupons. The range of variance of the values for the dcPP was also lower than the smooth coupon (Figure 4.6).

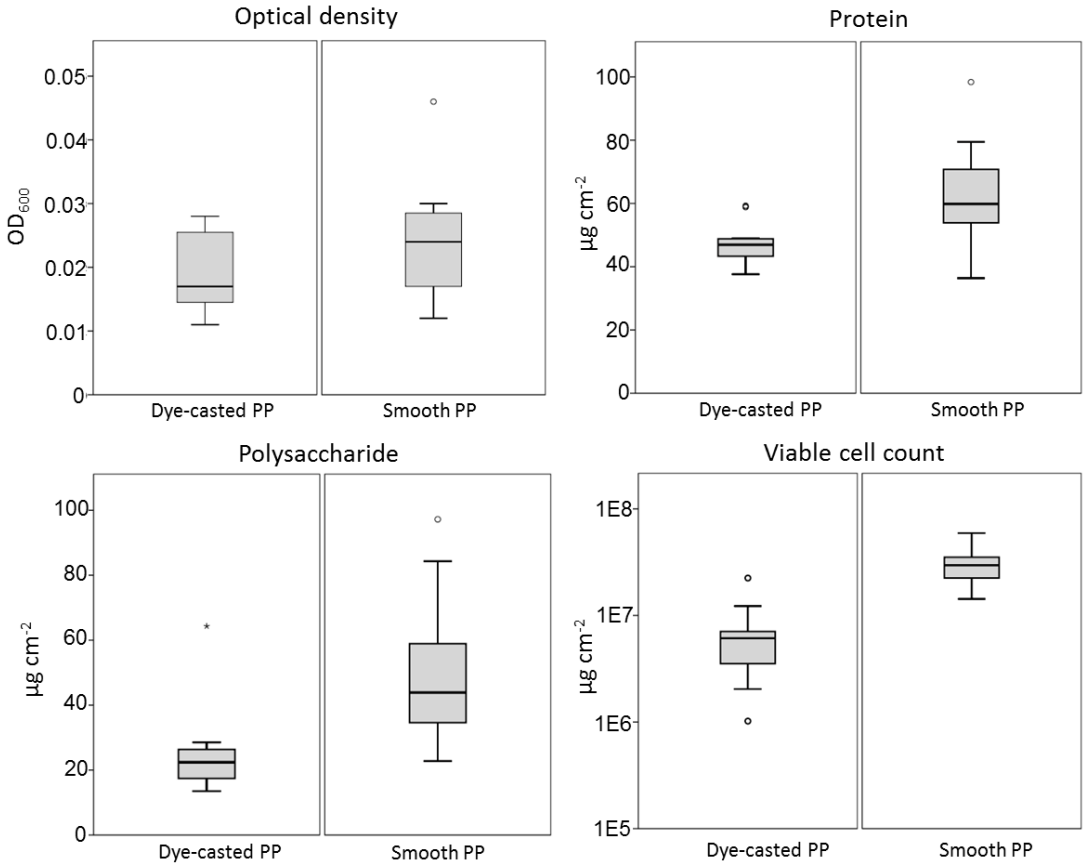


Figure 4.6. Box plot analysis of biofilm formed on smooth and dye-casted polypropylene (PP) for OD_{600}, protein, polysaccharide and viable cell count.

The difference of viable cell numbers between the dc and the smooth coupons were about one log (Figure 4.6). When the values are subdivided into the different holders the group median of the dc PP (except for OD_{600}) and the smooth coupons were relatively similar (Figure 4.7).

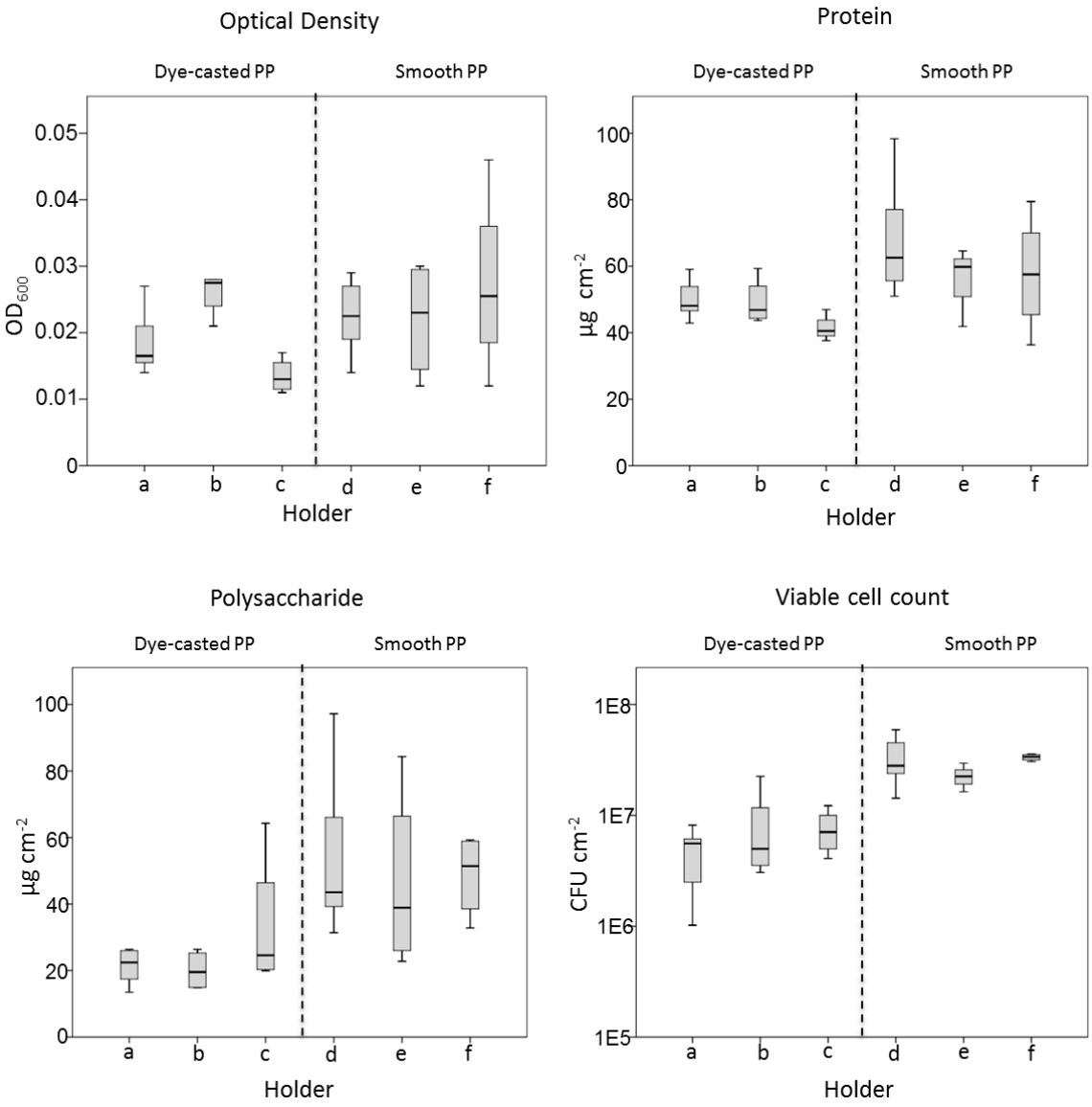


Figure 4.7. Box plot analysis for each quantified parameter of both tested materials subdivided into holders a, b, c for dye-casted PP and holders d, e, and f representing smooth PP.

Vertical alignment of holder a (dcPP) and holder d (smooth PP) showed that for the dcPP no vertical gradient could be determined for any of the tested parameters. For the smooth PP mainly for the viable cell counts, a significant linear relationship could be observed (Figure 4.8).

The tendency for a vertical gradient was reduced with the new experimental set-up C than with the experimental set-up A and B. This strongly supports the assumption, that the location of the medium feed and consequent distribution of the nutrients was resoponsible for spatial gradient formation. For the experimental set-ups A and B, the fresh medium entered the reactor through the surface of the culture broth and was distributed mainly by the quick cyclic pumping through the aeration loop and the vortices triggered by the rotation of the sample holders. In contrast to that, the experimental set-up D was modified in such a way, that the medium was supplied through a “needle” leading to the middle of the bioreactor (the entrance of the medium corresponding to the position No. 8 - 9 of the sample holders). This countermeasure decreased the formation of gradients. A signifcant gradient was only present for viable cell counts on smooth PP.

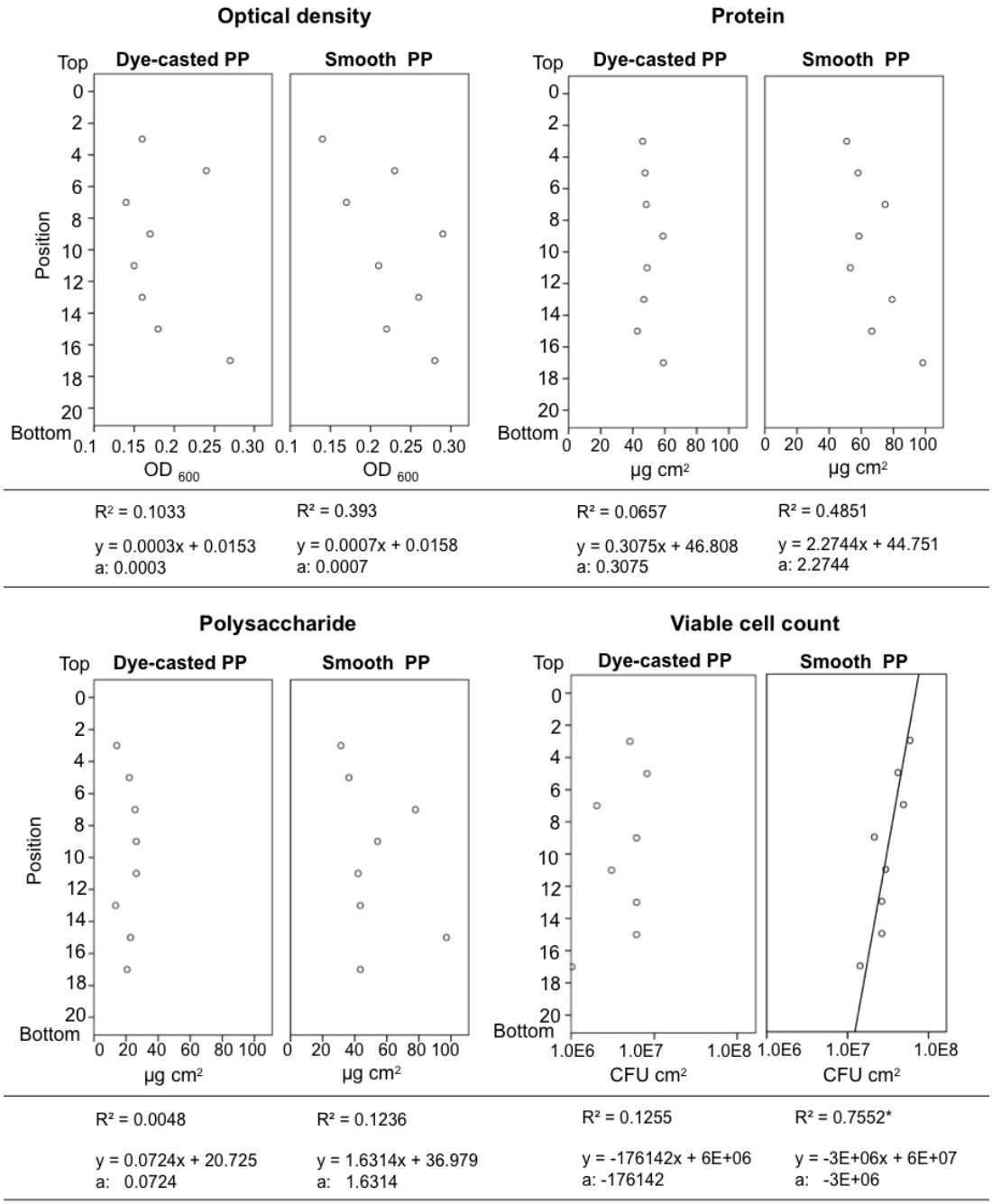


Figure 4.8. Vertical alignment of dye-casted PP (holder a) and smooth PP (holder d). Regression analysis was significant (*) with $\alpha = 0.05$.

4.3.2.5 Five independent reactor experiments with experimental set-up E: Repeatability of biofilm formation of *Pseudomonas putida* on dye-casted PP

For the assessment of repeatable formation of biofilms, *P. putida* was cultivated as previously described in minimal medium with glycerol as carbon source and growing only on dcPP coupons. The biofilms were harvested after 6 days p.i. and quantified. The repeatability of biofilm formation with dcPP was investigated. The experiments were conducted by other experimenters (Empa Testmaterials) to get also an insight into the reproducibility of the bioprocess. The experimental set-up D and set-up E were identical.

The biofilm formation has been quantified by determination of the amount of protein and polysaccharides and viable cell counts. Per experiment randomly chosen coupons (n = 3-6) were analyzed.

The biofilms tested for repeatability on dcPP coupons showed strong variation between the experiments especially for the amount of protein and polysaccharide. The largest range of variance of protein and polysaccharide was found in experiment No. 3 (see Figure 4.9). Interestingly, in experiment No. 2, the median for polysaccharides was the highest but was very low in protein content. In the remaining experiments (No. 1, 4 and 5), the protein content, the amount of polysaccharide, and the viable cell count correlated well with each other.

Although the five independent experiments were always performed in the same manner, a large difference between the experiments (e.g amount of proteins) was observed. The reasons for the large variability between the five independent experiments needs to be further clarified as several different parameters influence the cultivation process (e.g. fitness of the strain, nutrient vailability, material surface and experimenter). As these experiments with this set-up E were performed by other experimenters than in the previous experimental set-ups it is difficult to determine the main influence on the experimental outcome. One explanation is that the manner of execution can deviate from experimenter to experimenter leading to the observerd results and therefore, the protocol still needs to be improved to increase the repeatability and reproducibility of biofilm formation.

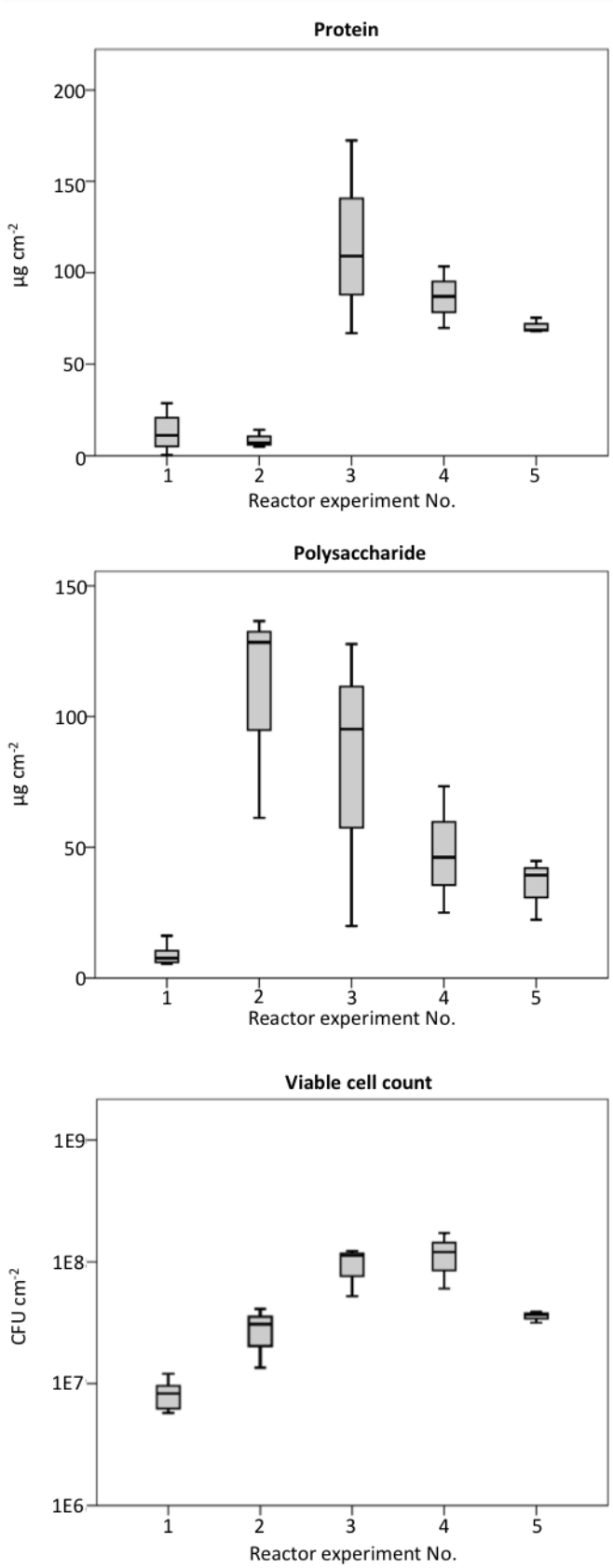


Figure 4.9. Box plot analysis protein (top), polysaccharide (middle) and viable cell count (bottom).

4.4 Conclusions

The ruggedness test matrix is a useful tool to reduce the amount of experiments analyzing the influence of seven nutrient upon biofilm formation. The information of nutrient consumption can be used for triggering the transition from planktonic to attached cell but also in the reversed sense to reduce fouling of surfaces. An example is the usage of iron chelating agents such as lactoferrin. Its high affinity for iron atoms stops biofilm formation (Stewart 2003). The control of biofilm formation by governing the nutrient availability is a way to reduce the amount of disinfection needed to reduce the amount of biofilm.

The designed biofilm minimal medium is supporting the formation of biofilms covering most of the essential nutrients that are needed. The minimal culture medium also allowed the cells to grow slower and in a more controlled manner.

The presented bioreactor setup, it was possible to produce biofilms on different types of polypropylene and also other materials. The smooth polypropylene used in washing machines was the most appropriate material because it allowed increased colonization of the surface. However, we were not able to produce highly repeatable biofilms of *P. putida* on smooth polypropylene coupons due to spatial gradient formation mainly due to the location of nutrient input. Nonetheless, biofilm formation is feasible and simple modifications (e.g. location of feed entry) could improve the spatial gradient within a reactor experiment.

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5. Evaluation of conventional preservation techniques for long-term storage of bacterial and yeast biofilms

Mauclaire L., Gattlen J., Kaiser J.P.[1], Amberg C.[2] and Zinn M.*

Laboratory for Biomaterials, Empa Swiss Federal Laboratories for Materials Science and Technology, Lerchenfeldstrasse 5, CH-9014 St. Gallen

[1] Materials-Biology Interactions Laboratory, Empa Swiss Federal Laboratories for Materials Science and Technology, Lerchenfeldstrasse 5, CH-9014 St.Gallen

[2] Empa Testmaterials AG, Moevenstrasse 12, CH-9015 St. Gallen

*Corresponding author:

Mailing address: Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland

Telephone: +41 58 765 76 98

Fax: +41 58 764 77 88

E-mail: manfred.zinn@empa.ch

Manuscript in preparation for submission to journal “International Biodeterioration and Biodegradation”.

My contribution was the cultivation of the biofilms and partial analysis of the biofilms. (Dubois assay).

Abstract

Biofilms are complex communities of microorganisms embedded in a cell secreted matrix attached to surfaces. The aim of this study was to identify the most appropriate strategy to preserve microbial biofilms for cleaning efficacy tests. Polypropylene coupons were supporting monospecific biofilm grown under continuous cultivation. Biofilms were constituted either by *Pseudomonas putida*, *Escherichia coli* or *Rhodotorula mucilaginosa*. The biofilms were stored at -80°C, -20°C, 4°C, and 20°C and eventually in presence of preserving agents. The biofilm remaining attached to the test coupons was evaluated after two weeks and two months of storage by quantifying exopolysaccharides, proteins, viable cells and resistance to removal. Freezing did not adequately preserve the biofilms even in presence of preserving agents. The most appropriate procedure for biofilm preservation was storage at 4°C under humid conditions, in presence of preserving agents. Whereas such procedures allowed an adequate preservation of protein and living cells (less than 1-log of reduction), the majority of the polysaccharides was destroyed after 2-months of storage. Nevertheless, it was possible, especially for *P. putida* stored at 4°C in trehalose, to preserve sufficient organic material and viable cells to enable removal tests with statistical significance.

Keywords: biofilm, storage, preservation, removal test

Highlights:

- Classical preservation methods such as freezing leads to biofilm desegragation
- Storage at 4°C under humid conditions, eventually in presence of trehalose or hydroxyectoine allowed an appropriate preservation of biofilm
- After two months of storage less than 1-log reduction of living cells was observed whereas the majority of polysaccharides were degraded
- The developed methodology was suitable to preserve bacterial biofilm of *P. putida*
- Yeast biofilm of *R. mucilaginosa* which comprised mainly polysaccharides could not be preserved with tested storage procedures

5.1 Introduction

Biofilms are the normal way of life of microorganisms to cope with environmental stresses such as shear force (Filoche et al. 2004), dehydration (Monier and Lindow 2003), starvation (Flemming and Wingender 2010), antimicrobial agents (Stewart 2002) or predation (e.g. Wey et al. 2008). In nature, the preserving agent is the cell auto-secreted matrix consisting of exopolymeric substances (EPS) which binds the cells together and to the surface (Flemming et al. 2007). The EPS comprise in particular polysaccharides (e.g. Laue et al. 2006) and therefore biofilms are pretty similar to preservation methods via encapsulation with organic polymer such as agar or alginate. In addition, it has to be considered that cells within biofilms are growing slowly which should increase their physiological adaptation to storage (Alpert 2005).

In industry, microorganims immobilized on surfaces are frequently used for food production (e.g. vinegar, Tesfaye et al. 2002), drinking- and wastewater treatments (Gros et al. 1988; Kim et al. 2004) and high-tech applications where cells need to fulfill sensory tasks, e.g. detection of heavy metal contamination of soil and aquifers (Verma and Singh 2005). Consequently, there is also a great interest to maintain functional cells that can be implemented on request.

The current industrial standard for microorganism preservation is freeze drying (e.g. Bjerketorp et al. 2006), which is used for culture collections and has a proven industrial performance record for planktonic cells. However, it is a costly and complex procedure and products are very sensitive to moisture. Alternative procedures are immobilisation and encapsulation in either inorganic (e.g. sol-gel, Fennouh et al. 2000) or organic polymers such as agar (Jouenne et al. 1994) or alginate (Prabakaran and Hotia 2008).

By genetic engineering or through controlled exposure to environmental stress, one can improve the preservation tolerance. For example increase of desiccation tolerance was observed for *Escherichia coli* containing endogenous compatible solutes such as trehalose (Tunnacliffe et al. 2001) or *Pseudomonas putida* engineered to produce hydroxyectoine (Manzanera 2002).

However, such genetic engineering tools are restricted in their range of applications because of tight regulations of usage. Current developments demonstrate the need for appropriate storage methodology, e.g. biofilm cells are needed for the development of biofilm sensors (e.g. Stocker et al. 2003; Struss et al. 2010), elaboration of biofilm removal

technologies (Gattlen et al. 2010) or as source of cells for membrane reactors and *in situ* bioremediation (e.g. Vogelsang et al. 1999; Laurin et al. 2006; Vlaeminck et al. 2007). The main bottleneck of all these applications is the procedure to preserve the biofilm. As a potential solution, we supposed that physiological state of biofilms might be used as a natural way to improve preservation of microorganisms. The preservation of biofilms has only been investigated poorly and to date there is a lack of knowhow to conserve the 3D-structure. For environmental samples, it is usually recommended to store biofilms at 4°C for a maximal duration of five days (e.g. Smucker et al. 2009) which certainly limits their utilisation.

The aim of this study was to develop a long-term storage procedure (up to two months) for bacterial and yeast biofilms preserving their unique properties because this is required for the development of a model biofilm as a sensor to quantify biofilm removal efficiency of detergents or washing processes. Of particular importance were the preservation of the biofilm cells with respect to the attachment to polypropylene surfaces, their amount of EPS and viability.

5.2 Materials and Methods

All chemicals were provided by Sigma-Aldrich/Fluka, Buchs Switzerland if not stated differently.

5.2.1 Biofilm formation

Biofilms were formed on coupons in a custom-made reactor as described elsewhere (Chapter 3). Polypropylene (PP) coupons were fixed on a stainless steel cylinder which was mounted on the stirrer axis of the reactor. *E. coli* PHL628 (curli) (Brombacher et al. 2006), *P. putida,* and *R. mucilaginos*a were used as test organisms representing Gram-negative bacteria and yeast strains (Vidal et al. 1998; Gattlen et al. 2010). Shake-flask cultures of cells in the late exponential growth phase were inoculated in a 3.7 L bioreactor. Cells were grown in minimal mineral medium containing 4 g L^{-1} glycerol for 13 and 6 days for bacteria and yeast, respectively (Chapter 3).

5.2.2 Preservation techniques

Coupons containing biofilms were sampled and rinsed in saline solution (0.9% NaCl w v^{-1} in water) to remove loosely attached cells. Each coupon was placed in individual Eppendorf tubes (2 mL) and stored according to the following procedures:

In a first set of experiments the possibility of freezing to store *E. coli* PHL628 biofilm was assessed. The biofilms were stored in Eppendorf tubes according to 8 procedures (Table 5.1). Results of storage experiments with *E. coli* PHL628 were used to simplify the protocol for long-term storage of *P. putida* and *R. mucilaginosa*: three temperatures were tested (-20°C, +4°C, +20°C) in combination with the following preserving agents: Tris-buffer (1 M), trehalose (0.1 M), hydroxyectoine (0.4 M) and glycerol (0.1 M). Concentrations of preserving agents were chosen according to the literature (e.g. Abadias et al. 2001; Gorman and Adley 2003; Manzanera et al. 2004), and biofilms without preserving agents were used as control.

All biofilms were examined two weeks and two months after the storage and compared with biofilm before storage without addition of preserving agents (time 0). At least three coupons for each preservation treatment were analysed for each sampling.

Table 5.1. Summary of the experiments conducted to preserve *E. coli* PHL628 biofilms. The biofilms were stored up to two months in Eppendorf tubes according to eight procedures.

	Protectant	**Concentration of protectant [M]**	**Treatment before storage**	**Storage temperature [°C]**
a)	none	-	dehydration in vacuum chamber	20
b)	glycerol	0.1	dehydration in desiccator	20
c)	none	-	none	4
d)	glycerol	0.1	none	4
e)	glycerol	0.1	4h on ice	-20
f)	glycerol	0.5	4h on ice	-20
g)	glycerol	0.1	rapid freezing in liquid nitrogen	-80
h)	glycerol	0.5	rapid freezing in liquid nitrogen	-80

5.2.3 Biofilm imaging, quantification and stress resistance

Biofilms were stained for microscopic observations before and after storage. Polysaccharides of the EPS were stained with Concanavalin-Alexa633 (final concentration 0.1 mg mL^{-1}, Molecular Probes, Invitrogen Lucerne, Switzerland) and DNA with Syto BC (Molecular probes; final concentration 0.5 µM) for at least 30 min (Neu 1992). Stained biofilms were examined with a confocal laser scanning microscope (CLSM Axioplan 2 Imaging LSM510, Zeiss, Feldbach, Switzerland). The samples were excited at 632 and 488 nm for Alexa633 and Syto BC, respectively. Images were acquired and treated with the software LSM Image Examiner (version 4.0.0.241, Zeiss).

For biofilm quantification, coupons were firstly rinsed in saline solution (0.9% w v^{-1} NaCl) to remove loosely attached cells and preserving agent, transferred into tubes containing NaCl solution (5 mL) and biofilm was detached by sonication (power 10%, 50% active cycle, 1 min Branson sonifier, Carouge, Switzerland). An aliquot (0.5 mL) was taken for protein quantification using micro BCA kit (Thermo Scientific, Rockford Illinois, U.S.A.) according to the manufacturer's description. Protein content was expressed as mg of bovine serum albumin (BSA; Sigma-Aldrich/Fluka, Buchs, Switzerland) equivalent per cm^2 of PP coupon. An aliquot (2 mL) was taken for polysaccharide quantification based on the Dubois method (Dubois et al. 1956). The supernatant was mixed with 0.05 mL of 80% phenol solution (Fluka) and 5 mL of 98% sulphuric acid (Merck, Zug, Switzerland), letting stand for 10 min to cool down and subsequently incubated in a water bath at 26°C ± 1°C for 20 min. Samples were vortexed for 1 min before measurement of OD at 485 nm. Polysaccharide content was expressed as mg of glucose equivalent per cm^2 of coupon. An aliquot (0.5 mL) was taken for determination of viable cell counts by 10-fold dilution series and plating on tryptic soy agar and Sabouraud dextrose agar for bacteria and yeast, respectively. Viable cell counts were expressed as colony forming units per cm^2 of coupon.

Adhesion to the surface of the biofilm after storage was examined by a removal assay. Briefly, coupons stored for two weeks were fixed at the bottom of a 1 L glass beaker and submerged in water (0.5 L) containing a standard detergent (per L: 5.39 g IEC-A base, 1.4 g sodium perborate and 0.21 g tetraacetylethlyenediamine) (IEC/SC 59D Home Laundry appliances, 2010). Beakers were incubated in a shaker (120 rpm) at 30°C for 25 min. The

coupons were then detached, rinsed three times with filter sterilized water (0.1 L). The remaining biofilm cells were detached and quantified by crystal violet staining (0.1% w v^{-1} for 20 min) (Merritt et al. 1998). Coupons were rinsed and colour intensity was evaluated using a spectrophotometer (Konica Minolta CM-2600d, Konica Minolta Sensing, Inc., Japan) according to the CIE Lab colour scale (CIE 1976 L*a*b*), where each colour is defined in a 3D colour space with the three parameters L* (lightness), a* and b* where L*=100 is white and L* = 0 black, and a* (-a = green, +a = red) and b* (-b = blue, +b = yellow). The Euclidian distance between the points on the Lab scale before and after removal indicated the change of colour intensity and was proportional to the amount of biofilm that got removed during the assay. The colour intensity was estimated by averaging measurements conducted on three locations per coupon and removal tests were conducted on at least four coupons.

5.3 Results

The ability to store biofilms and preserve their typical properties are the most important steps for development of test biofilms to be used in the evaluation of hygienic treatments. In this study, the storage performance of Gram-negative bacteria *E. coli* PHL628 and *P. putida*, as well as the yeast *R. mucilaginosa* were tested.

5.3.1 Preservation of *E. coli* biofilms

For an initial storage assay, *E. coli* PHL628 was chosen being a good biofilm forming strain producing a lot of exopolymeric substances. A first set of experiments was conducted to evaluate the influence of temperature of storage in combination with the most commonly used preservative, glycerol. Figure 5.1 shows the amount of protein retrieved from *E. coli* biofilms before, after two weeks, and two months of storage period. Freezing, in presence of 0.1 and 0.5 M glycerol, was an appropriate procedure to preserve biofilms, neither at -20 nor at -80°C. Glycerol by itself was not pernicious for preservation since it had no significant influence on biofilms stored in fridge or at room temperature with respect to the amount of protein. The preservation during short-termed storage (two weeks) in the fridge under humid conditions seemed to be the most appropriate method, whereas for longer storage (two months) dehydration and storage at room temperature

appeared as a valid option (Figure 5.1). Using the latter procedure the variability between the replicates increased but after an initial loss, the amount of living cells in the remaining biofilm became stable over the two months of storage.

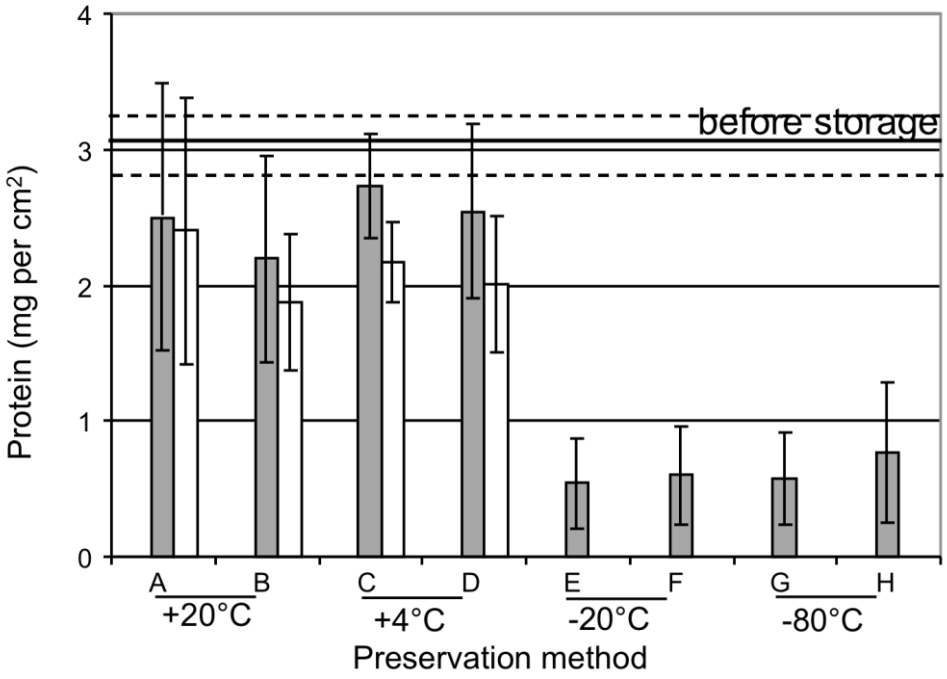

Figure 5.1. Amount of proteins retrieved from *E. coli* PHL628 biofilm. Grey bars: value after two weeks of storage, white bars: values after two months of storage. The biofilms were treated according to an experimental plan listed in Table 5.1. Bars represent average value ± standard error (n = 3).

5.3.2 Preservation of *P. putida* biofilms

Based on the results obtained with preservation of *E. coli* PHL628 biofilms, a second set of experiments was designed to use alternative preservatives to glycerol. It was tested whether other cryoprotective agents preserved the biofilms better with reduced losses of organic matter after two weeks and two months. However, *E. coli* PHL628 (curli) was an engineered organism, it was not considered for further testing, while the wild-type *P. putida*, is a widely distributed microorganism that can be isolated from different natural and man-made systems.

Figure 5.2 showed the amount of *P. putida* biofilm quantified by living cells, total proteins and total polysaccharides before and after storage. As already observed with *E. coli*

PHL628, freezing did not allow an appropriate preservation of the *P. putida* biofilms. Considering the overall parameters describing the biofilms, the most appropriate method to preserve the *P. putida* biofilms appeared to be the storage at 4°C in presence of hydroxyectoine or glycerol. Under these two conditions, we observed about 50% reduction of the polysaccharide content, a 25% reduction of the protein content, and less than 1-log reduction of living cells after two months of storage (Figure 5.2).

The adhesion of 2 weeks-stored biofilms to PP was characterized by the removal assay. Figure 5.3 summarizes the intensity of crystal violet staining on Lab scale for stored biofilm before and after removal test. The colour measurements indicated that the Euclidian distance was maximal for biofilms stored at 4°C in presence of trehalose and glycerol (35.1 and 27.1, respectively) compared to biofilms stored at room temperature with same preservatives (7 and 9.9, respectively). These results indicated that *P. putida* biofilms stored at room temperature have lost a large part of their organic matter (e.g. polysaccharide and protein), which is required for visual examination with crystal violet, already before the removal test. This observation is in agreement with quantitative results obtained for polysaccharides, proteins and living cells (Figure 5.2).

On the other hand, storage at 4°C in presence of glycerol or trehalose was appropriate to allow discrimination by removal test. Under these conditions a higher amount initial biomass was present after storage permitting a better differentiation before and after the removal test.

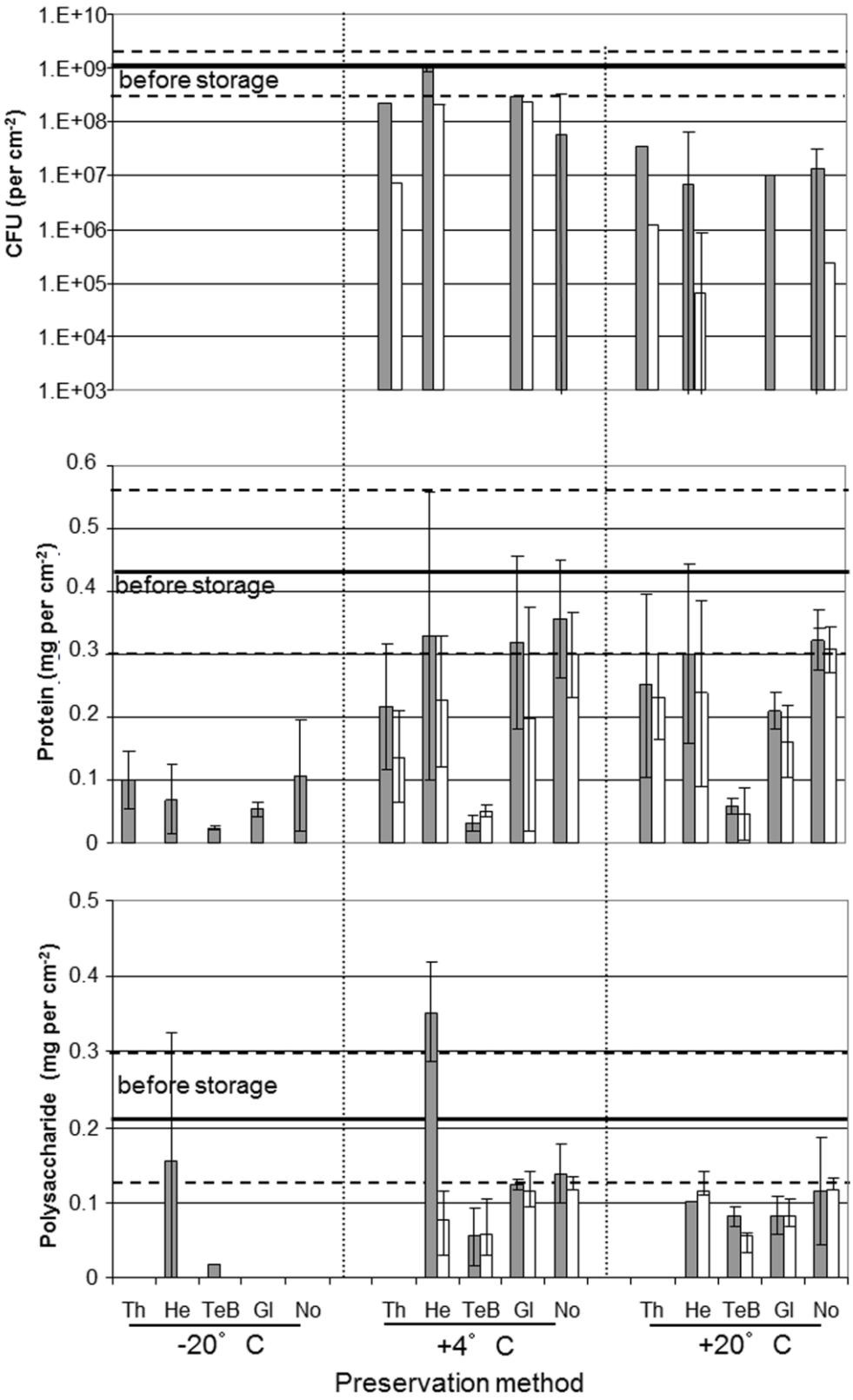

Figure 5.2. Amount of living cells (colony forming units), proteins and polysaccharides retrieved from *P. putida* biofilm. Grey bars: value after two weeks of storage, white bars: values after two months of storage. The biofilms were stored in trehalose (Th), hydroxyectoine (He), Tris-buffer (TRB), glycerol (Gl) or without protectant (No) at -20, +4 and +20°C. Bars represent average values ± standard errors (n = 3).

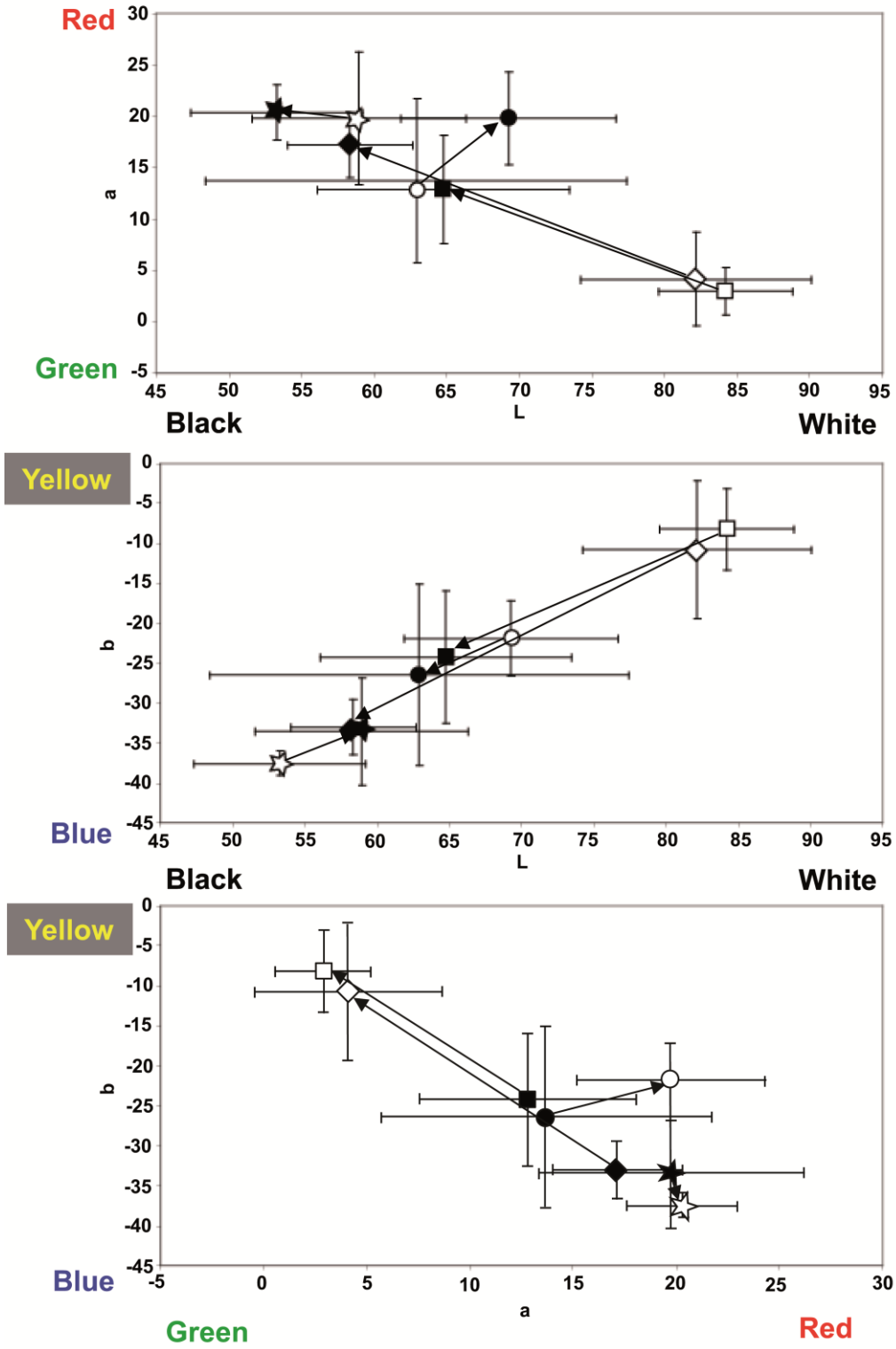

Figure 5.3. Colour intensity of crystal violet staining before (black symbols) and after (empty symbols) removal test. The biofilms were stored in trehalose at 4°C (diamond) and 20°C (star) or in glycerol at 4°C (square) and 20°C (circle) for two weeks. The arrow indicates the Euclidian distance of the colour change before and after removal test.

5.3.3 Preservation of *R. mucilaginosa* biofilms

Yeast biofilms are a raising problem in the medical field, in the food industry or in general terms of hygiene. But in contrast to bacterial biofilms, yeast biofilms are still relatively unexplored regarding its formation and preservation.

Figure 5.4 shows the number of living cells and the amounts of proteins and polysaccharides retrieved from *P. putida* biofilms before and after storage. In analogy to the results obtained with bacterial biofilms during storage at 4°C, this procedure appeared to be the most appropriate method to preserve *R. mucilaginosa* biofilms. However, in contrast to bacterial biofilms, the addition of preservatives did not significantly improve cell viability during storage for 2 months. When kept at 4°C under humid conditions the *R. mucilaginosa* biofilms lost about 1 log of living cells whereas protein content after an initial increase remained stable. As we observed previously with bacterial biofilms, the polysaccharides were largely destroyed during the storage and only about 10% of the polysaccharides initially present remained detectable after two months of storage.

Preservation of biofilm was determined by confocal microscopy observations (Figure 5.5) using criteria such as biofilm thickness, cell integrity and colocalisation of EPS. The latter one was identified by the yellow colour due to overlay of cells stained in green and EPS stained in red. Micrographs confirmed results of biofilm quantification (Figure 5.4). In particular, freezing removed a majority of the biofilm with respect to cells and EPS, even in presence of preserving agent (Figure 5.5, left column). Hydroxyectoine appeared to be more appropriate to preserve biofilm at 4°C. However, a closer look at the micrographs revealed that the cells were disrupted during storage. Nevertheless, the most appropriate temperature to store biofilm appeared to be 4°C. At this temperature, presence of trehalose and hydroxyectoine was concluded to be beneficial on the preservation of EPS. Tris-buffer did not allow any appropriate preservation of the *R. mucilaginosa* biofilm for all tested temperatures.

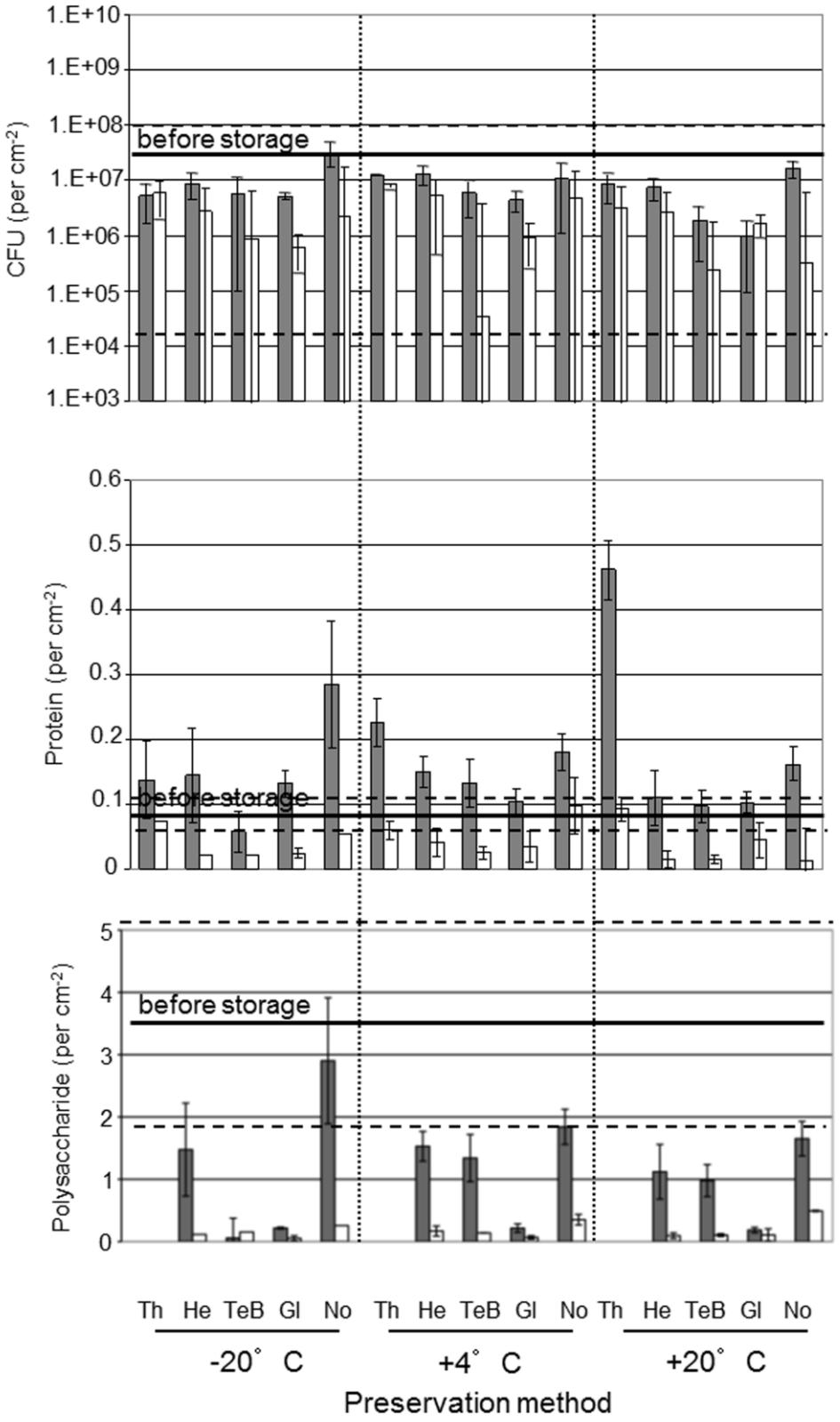

Figure 5.4. Amount of living cells (colony forming unit), proteins and polysaccharides retrieved from *R. mucilagonosa* biofilm. Grey bars: value after two weeks of storage, white bars: values after two months of storage. The biofilms were stored in trehalose (Th), hydroxyectoine (He), Tris-buffer (TeB), glycerol (Gl) or without protectant (No) at -20, +4 and +20°C. Bars represent average values ± standard errors (n = 3).

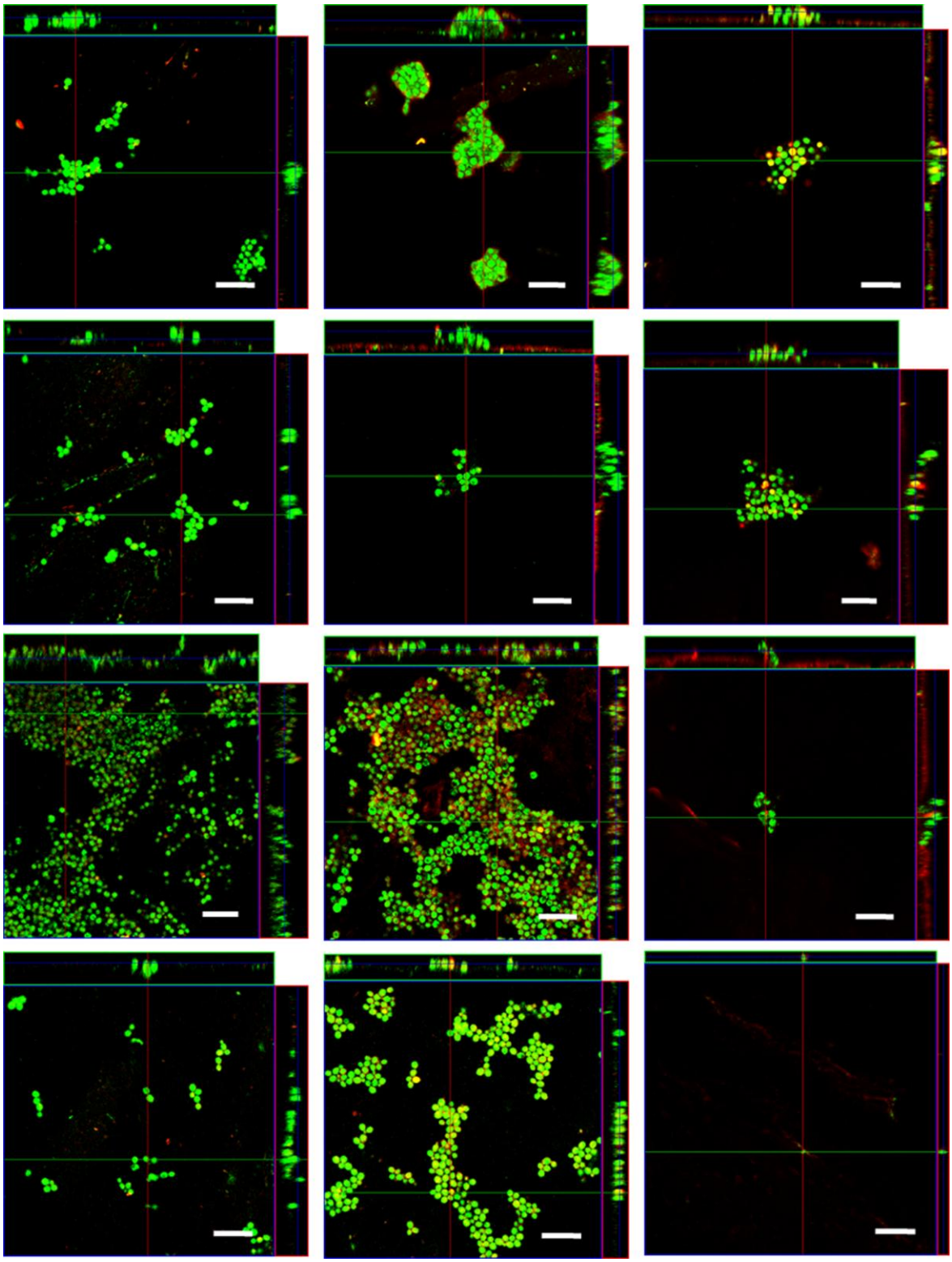

Figure 5.5. Confocal images of *R. mucilaginosa* biofilms after two weeks of storage. Storage temperatures were -20°C (left panel), 4°C (center panel) and +20°C (right panel). Biofilms were stored without preserving agent, in trehalose, in hydroxyectoine and in Tris-buffer (from top to bottom, respectively). Scale bar = 20 µm.

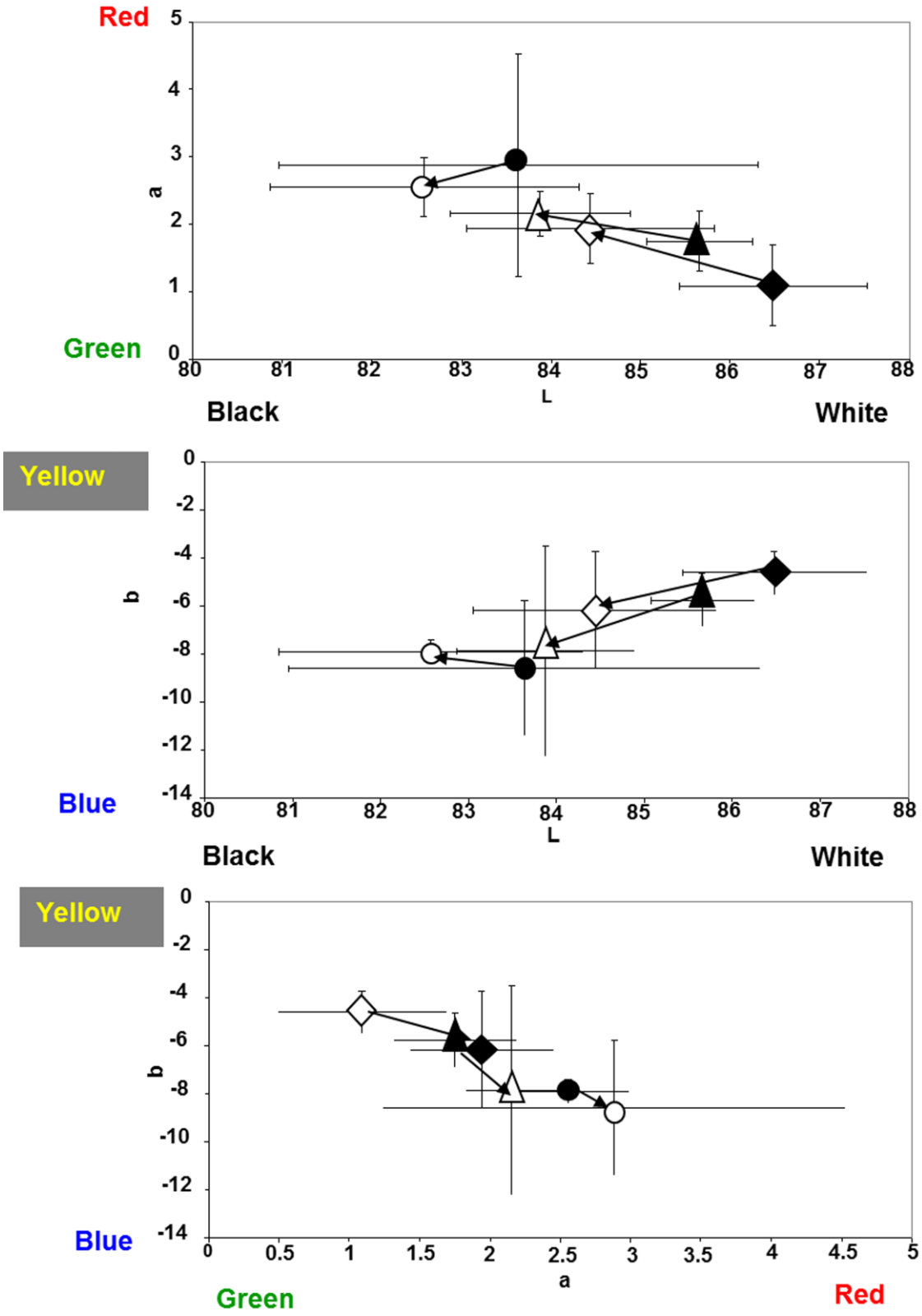

Figure 5.6. Colour intensity of crystal violet staining before (black symbols) and after (empty symbols) removal test. The biofilms were stored in glycerol (triangle), trehalose (diamond) or without preserving agent (circle) at 4°C for two weeks. The arrow indicates the Euclidian distance of the colour change before and after removal test.

The adhesion of yeast biofilms stored at 4°C for two weeks was evaluated by the removal assay (Figure 5.6). The Euclidian distance of crystal violet staining before and after removal test was 1.3, 2.8 and 2.7 for biofilms kept without preservative, in glycerol, and in trehalose, respectively. These results indicated that none of the three investigated storage conditions enabled an appropriate preservation of biofilm. Despite the fact that the living cells and protein contents were not significantly influenced during two weeks of storage (Figure 5.4), it was not possible to differentiate biofilms before and after the removal test because of very low initial values of biofilm content. This observation indicated that most of the *R. mucilaginosa* biofilms were removed during the storage procedure already before the removal test and is in agreement with the massive losses of polysaccharides observed during storage (Figure 5.4), polysaccharides representing about 90% w w^{-1} of the dry biomass of *R. mucilaginosa* biofilms.

5.4 Discussion

Microbial cells living in biofilm or in planktonic state have different appearance, genetic expression, metabolism, and physiology (Prakash et al. 2003; Kives et al. 2006). Therefore, it is not surprising that conventional procedures to preserve planktonic cells, like freezing or drying, were not completely adapted to biofilms. In particular, in this study, we demonstrated that storage below the freezing point led to the destruction of the biofilm matrix. During freezing the specific density of water is changing and therefore the 3D-network of the EPS might be torn apart. After thawing the biofilm got disintegrated and detached easily from the substratum material. Interestingly, none of the tested protective agents was sufficient to preserve the biofilm matrix from freeze-thawing stresses. Preservation of biofilm is a controversial subject and previous studies on the conservation of aerobic and anaerobic biofilms showed divergent results. On one hand, Vogelsang and collaborators (1999) recommended freezing without additives as preservation technique for the long-term storage of nitrifying biofilms. Whereas Laurin and collaborators (2006) studying preservation of denitrifying biofilms up to 17 months insisted on the addition of an appropriate cryoprotectant - in this particular case glycerol - for a perfect conservation of denitrification activity and microbial diversity after freezing at -20 and -80°C. On the other hand, Vlaeminck and collaborators (2007) recommended long-term storage at 4°C rather than freezing, a procedure which is similar to what we

found to be the most appropriate for the storage of *E. coli* PHL628 (curli) (Brombacher et al. 2006), *P. putida*, and *R. mucilaginosa* biofilms.

Despite the fact that different species of microorganisms exhibited a large variability of preservation results with respect to storage conditions (Heckly 1978; Donev 2001; Bjerketorp et al. 2006), we found that the most appropriate procedure to preserve our bacterial and yeast biofilms was storage at low temperature (4°C), under humid conditions and eventually in presence of hydroxyectoine, glycerol or trehalose. Under these conditions, the losses of living cells and proteins were limited even over a 2-month period. However, the main problem of biofilm preservation is the preservation of the EPS matrix which holds cells together and fixes them to the supporting surface. This matrix is mainly constituted by polysaccharides (Sutherland 2001) - and with respect to shelf-life of polysaccharides - the investigated procedures were by far not perfect. In the case of *P. putida*, where polysaccharides represented about a third of the dry biofilm biomass, the "best" procedure allowed to preserve 50% of the polysaccharides. It was found that the majority of the loss already occurred in the first two weeks of storage. Despite this major loss, enough biofilm remained to be stained and quantified with crystal violet for subsequent washing tests. In the case of the yeast *R. mucilaginosa*, where polysaccharides represented 90% of the dry biofilm biomass, the "best" procedure enabled preservation of only 10% of the polysaccharides originally present. The majority of this loss occurred between two weeks and two months of storage at 4°C. However, already after two weeks the remaining amount of biofilm was insufficient to be used as indicator biofilm for removal tests.

5.4.1 Factors influencing the preservation of biofilms

In presence of an appropriate preserving agent the reduction of living cells was limited to 1-log whereas without such agent nearly all the cells died during storage for two months.

Based on our results the question, whether an initially large content of polysaccahrides within the EPS contributed to the preservation of cells remained still open. Typically, the use of preserving agents is recommended to improve cell survival below the freezing point (Donev 2001). However, in our experiments we observed that the addition of preserving agents was also beneficial for storage above 0°C. Thus, they showed a positive impact on preservation of living cells, but only a limited benefit on the preservation of the protein

and polysaccharide contents of *P. putida* and *R. mucilaginosa* biofilms. For these two microorganisms, the most appropriate storage procedure was 4°C under humid conditions which is similar to a classical storage procedure developed for testing sanitizing agents against food-borne biofilms. Somers and Wong (2004) demonstrated that survival of *Listeria monocytogenes* biofilm was higher during 5-day storage at 4°C than at 10°C and that the presence of food residues increased the survival percentage by 7%. In our study, we demonstrated that the duration of storage could be extended from few days (classical test for food pathogens) to two months. However, prolonged storage durations might influence besides the biofilm composition (EPS and cells) also its structure/architecture significantly. For example *Listeria monocytogenes* showed an increased resistance against various sanitizing agents after 14 days of storage at 15°C (Stopforth et al. 2002). Similarly, the frequency of resting cells (e.g. persisters) of *Pseudomonas aurantiaca* and *P. fluorescens* increased with storage time (Mulyukin et al. 2008). The possibility to add alginate to the biofilm before adding the preserving agent was not a valid option for our purpose. This approach would include an additional step and could reduce the outcome. Moreover, it was also desired to keep the biofilm in its natural form. In analogy, genetical engineering of strains in order to enhance storage performance was not considered as an option, although this could improve the stress resistance of the microorganism by increased production of intracellular or extracellular preservation compounds. However, genetically engineered organisms cannot be applied unrestrictedly and their usage outside the laboratory is under strict regulations.

5.5 Conclusions

It was found that the regular preservation methods used for planktonic cells cannot be applied to maintain the typical properties of biofilm cells (3D-structure and attachment). In our hands, the most appropriate procedure to preserve bacterial and yeast biofilms was the storage at 4°C under saturated humid atmosphere eventually in presence of preserving agents (e.g. trehalose or hydroxyectoine). This storage procedure was adequate to maintain both cell viability and a sufficient integrity of the *E. coli* PHL628 and *P. putida* biofilms which could be used as indicator of biomass) for removal tests of up to at least 2-weeks. However, because this procedure poorly protected the polysaccharide matrix, it was insufficient to maintain the integrity of *R. mucilaginosa* biofilms.

Acknowledgments

This work was granted by CTI (AO-LS-99-MAP-LSS-018). The authors would like to thank Bettina Lanz, Monika Braegger and Kathrin Grieder for their technical support.

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6. From a washing machine isolate to a model biofilm for testing biofilm removal in household washing machines

Abstract

No reliable test system is available to quantify the cleaning and removal efficiency of biofilms in household systems such as washing machines. Therefore, model biofilms with *Pseudomonas putida* and *Rhodotorula mucilaginosa* were produced to use them as reference for the determination of washing efficiency of household washing machines. Different dyes were tested to determine biofilm formation. The biofilms together with a staining procedure (with crystal violet) are the main part of the biofilm removal kit. The biofilms were tested for stability in terms of storage, transport and real-life washing. For storage and transport of the produced biofilm trehalose was the most appropriate solute to keep biofilm, especially of *R. mucilaginosa*, intact. Low washing temperatures and bleach-containing detergents were not able to remove the entire biofilm. The biofilms were sufficiently stable to test real-life conditions of washing machines and were able to serve as reference for cleaning efficiency of household washing machines.

Keywords: test system, single-species biofilms, biofilm removal

My contribution was the screening and production of biofilms.
Caroline Amberg performed the colorimetric and material studies and Dr. Laurie Mauclaire did the material analysis.

6.1 Introduction

Washing machines are typical water systems that are prone to biofilm formation. This problem became evident as the washing behavior started to change in order to fulfill the recommendations of ecologically friendly washing techniques. Low-temperature washing (30°C or lower) and increased usage of bleach-free detergents enhanced biofilm formation in washing machines.

Biofilm formation can impair the hygienic performance of the washing machine (Terpstra 1998), constantly contaminate the garments, lead to malodor formation (Munk et al. 2001) and contribute to corrosion (Little et al. 1991). Remaining, standing water with released nutrients from dirt (Szewzyk et al. 2000) and washing detergents (Okpokwasili et al. 1991) can nourish the cells in the washing machine.

Various manufacturers of household washing machines try to integrate systems to prevent and/or combat formed biofilms. Although several methods (e.g. steam, release of silver ions, ultrasound) are available to remove biofilms, no test method is available to determine the removal efficiency in household washing machines.

A part of the solution is the quantification of the biofilm. Different semi-quantitative methods are available that can detect biofilm formation. Most of these methods are based on staining dyes like crystal violet or methylene blue (Peeters et al. 2008) or fluorescent dyes like calcofluor white (Shih and Huang 2002) that are rather unspecific or interacting with the EPS of a biofilm. These semi-quantitative methods enable a fast and easy detection of biofilm removal efficiency of washing cycle, washing detergent or other parameters.

Our goal was to develop a standardized biofilm to determine the washing efficiency of household washing machines and the influence of temperature, mechanical forces as well as detergents on 2-weeks stored bacterial and yeast biofilms.

6.2 Materials and Methods

6.2.1 Washing machine analysis

Household washing machines and parts of them from four countries (USA, Korea, Germany and Switzerland) were analyzed with crystal violet (CV) for the existence and location of biofilm formation. The household washing machines were dismantled prior to

the staining procedure. The biofilms were sampled with swabs, isolated and identified as described in Chapter 2 (Gattlen et al. 2010).

6.2.2 Organisms

Cultivation experiments in different culture media and screening for biofilm formation have been conducted to find the most appropriate strains for biofilm production. Growth tests with the washing machine isolates have been conducted in Erlenmeyer shake flasks (see supplementary data Chapter 2). Although all tested organisms were able to grow under the defined conditions, not all were appropriate for further testing due to floc formation, slow or poor growth. Thus, three representative biofilm forming strains had been selected: the Gram-positive *Microbacterium* sp., the Gram-negative *Pseudomonas putida* and the eukaryotic *Rhodotorula mucilaginosa*.

6.2.3 Production of a single species model biofilm with *Microbacterium* sp., *P. putida and R. mucilaginosa* in a biofilm reactor

A frozen stock culture of *Microbacterium* sp. (1.8 mL) was transferred into 150 mL nutrient broth supplemented with 4 g L^{-1} glycerol. The cells were cultivated at 30°C, 150 rpm for 9.5 h or until an OD_{600} of 1.8 was reached. The suspension was transferred into the reactor (30% nutrient broth with 4 g L^{-1} glycerol) and the cells were grown until μ_{max} (ca. 0.37 h^{-1}) was reached (5 h). Thirty minutes later the wash out of cells was initiated with increasing the dilution rate to 0.41 h^{-1}. The dilution rate was lowered to 0.1 h^{-1} after ca. 41 h. After 14 days post inoculation (p.i.) the biofilms were harvested and quantified. The cultivation conditions for *R. mucilaginosa* were described in Chapter 3 and for *P. putida* in Chapter 4.

For staining tests, *P. putida* was cultivated in bioreactors (E2-medium, Chapter 2) to form biofilm on the material coupons. Metal, rubber and polypropylene were chosen as support material for biofilm formation and were cut into small test coupons.

6.2.4 Staining test

In order to evaluate the which stain works well, fluorescent and non-fluorescent dyes were tested: carbol fuchsin, congo red, CV, eosine, erythrosin B, fluorescein sodium, iodine, methylene blue, neutral red, rose bengale, safranine and the safranine kit.

6.2.5 Colorimetric measurements

A colorimeter (Konika Minolta CM-2600d) was used for quantitative color measurements. The principle of the colorimeter is to measure the amount of a* and b* (green/red, yellow/blue color) and the color intensity (white/black). The result of a color measurement is a vector defined by the L*a*b color space that contains all the visible colors (Figure 6.1). L* refers to the intensity, whereas L* = 100 is white and L* = 0 is black. + a* is the red, -a* is the green, +b* is the yellow and –b* is the blue channel. For the colorimetric measurement three locations on each coupon were averaged.
To determine the amount of biofilm the Dubois assay and the BCA kit assay were conducted and the cell number was quantified by serial dilutions and plating (as described in Chapter 2).

6.2.6 Shelf-life of biofilms

To be able to use the produced biofilms at any time point after the production for subsequent tests, the shelf-life of biofilms was assessed. Biofilms of *P. putida* and *R. mucilaginosa,* produced in the bioreactor were stored under different conditions using typical cryo protective solutions (e.g. hydroxyectoine) as described in Chapter 5. The amount of remaining biofilm was quantified after two weeks and two months.

6.2.7 *In situ* washing tests of stored biofilms

Coupons, that contained mature biofilm, had been stored for two weeks under different conditions. The coupons were implemented into a custom-made port (Wäschereitechnik Gugelman AG, Switzerland) on the outer side of the washing drum (upper backside, middle and lower position). The three locations for biofilm implementation have been chosen because they are exposured to different mechanical and chemical forces (Table 6.1).

Table 6.1. Distribution and intensity of chemical and mechanical stress at the different sampling locations in the washing machines.

Position in around washing drum	Chemical stress	Mechanical stress
Upper backside	high	moderate
Middle	moderate	moderate
Lower position	moderate	high

Four coupons were inserted per holder (one blank and three samples). The coupons of *P. putida* and *R. mucilaginosa* were harvested after the washing process a) without detergent, b) with the standard detergent IEC-A* or c) with a color gel detergent (Persil Color Gel^{TM}) at 30°C, and 40°C. In a second set of experiment coupons were exposed to Persil Color Gel^{TM}, washing at 20°C and 40°C. The removal of biofilm was quantified as removal of colony forming units.

6.2.8 Simulated transport conditions of biofilm coupons

Because model biofilms aimed to be used outside the laboratory, the fully mounted biofilm platform has to be stable during transport. Experiments were designed to simulate the transport conditions. Stored biofilms (no protectant at 4°C and 20°C, trehalose at 4°C and 20°C) were tested for their stability during transport. One coupon with *P. putida* and one with *R. mucilaginosa* was separated by two blank coupons. The carriers were vertically positioned into sterile Greiner tubes. The tubes were filled with a solution (trehalose, 0.9% saline solution and phosphate buffer) or without solution. The tubes were placed in a shaker at 30°C for 48 h to simulate the transport conditions by post/mail. The biofilms were exposed to three different conditions: a) no shaking, b) low shaking (a constant shaking alternating with a short vigorous shaking) and c) high shaking forces (100 rpm).

6.3 Results and Discussion

6.3.1 Location of biofilm formation

Household washing machines from four different continents were evaluated for the presence of biofilm forming microorganisms. Over 90 strains were identified covering Gram-negative, Gram-positive bacteria, as well as yeasts and yeast-like fungi (Gattlen et al. 2010). The main hotspots for biofilm formation were the drum, the detergent drawer or the filter. A screening with the 17 most commonly found strains was conducted to study their behavior in complete and minimal media (see supplementary data of Chapter 2). Some of the strains could not be considered for further testing, because either they were not able to be re-cultivated, had a long lag-phase, were growing slowly or formed flocs. Additional strains were tested from the washing machine to perform a comparative study of 22 strains with strains from the German strain collection (Gattlen et al. 2010).

The most appropriate strain for single-species biofilm formation in a benchtop reactor is a class I strain, that can easily be cultivated in a reproducible manner in terms of protein and polysaccharide amount and viable cell count, producing sufficient EPS for the biofilm detection and removal testing and remains stable during storage. Such biofilms can be shipped without any restrictions. For the production of biofilms, *P. putida* and *R. mucilaginosa* were considered for the cultivation of biofilms in a bench-top reactor for the representation of Gram-negative bacteria and yeast. For the Gram-positive bacteria, *Microbacterium* sp. was a promising candidate.

6.3.2 The choice of biofilm support and coloring test

A very important issue for this study was to choose the right support for biofilm formation. The material had to fulfill several requirements: ideally it is a material of the washing machine that is prone to biofilm formation, having a light color, being autoclavable, and showing no or low autofluorescence.

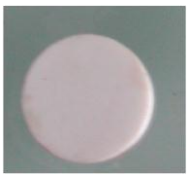
Polypropylene covered most of the material requirements like autoclavability or light material color. Autofluorescence of PP was the biggest problem, however, staining the cells with DAPI still allowed to differentiate the cells from the background.

The different materials with on-grown biofilms were exposed to different unspecific dyes that are commonly used to stain cell components. The material itself has to give sufficient contrast and show a clear difference between a surface that is not colonized and a surface

with biofilm. For example, on black rubber, the difference in color between with and without biofilm is very low. Also stainless steel absorbed most of the staining even withouth biofilm formation (Day 0) and therefore, did not give too much contrast before and after fouling (Table 6.2). In contrast to that, polypropylene gave the largest difference in color intensity before and after staining with all tested dyes. Crystal violet is a commonly used dye to detect biofilm as it interacts with negatively charged molecules (matrix and cell components).

Based on this knowledge, a color chart has been developed based on the CFU cm^{-2} of *P. putida* biofilms on the test coupon. The differences between the different quantities of CFU cm^{-2} and biofilm, respectively, was the greatest for materials with a white or grey/metallic background. Materials with a darker background did not, or only to smaller extent, exhibit different color intensities, which made a semi-quantitative measurement more difficult (Figure 6.1).

Table 6.2. The different dyes and their effect on coloring biofilms of *P. putida* grown on polypropylene, rubber, and metal for up to 12 days.

Material	Stain	Day 0	Day 5	Day 12 (or 10*)
Plastic 	**Carbol fuchsine**			
	Crystal violet			
	Methylene blue			*
	Safranine			
	Safranine kit			
Rubber 	**Crystal violet**			
	Methylene blue			
Metal 	**Safranine kit**			
	Carbol fuchsine			
	Crystal violet			

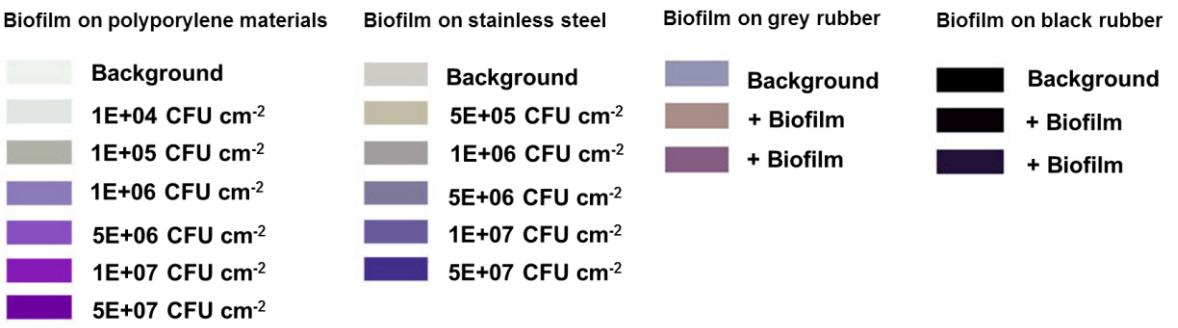


Figure 6.1. Color chart of polypropylene, stainless steel, grey and black rubber based on CFU cm^{-2}. The color intensity (after crystal violet staining) is correlating with the amount of present *P. putida*. The lighter/whiter the background, the better the different shades are visible.

6.3.3 Production of a single species model biofilm with *Microbacterium* sp., *P. putida* and *R. mucilaginosa* in bioreactors with round coupons

Biofilm formation of *R. mucilaginosa* and *P. putida* on polypropylene coupons were described in Chapter 3 and Chapter 4, respectively. The produced biofilms were used for storage and washing tests. The Gram-positive strain *Microbacterium* sp. cultivated in the reactor was growing relatively slow compared to *P. putida* or *R. mucilaginosa* and formed heterogeneous, filamentous biofilms. The mean amount of polysaccharides was 287 µg cm^{-2}, 504 µg cm^{-2} for proteins and $3.7x10^{9}$ CFU cm^{-2} for viable cells. The mean biomass was sufficient to be considered for removal studies. However, the biofilm formation of *Microbacterium* sp. was strongly impacted by shear forces therefore, the biofilm was mainly growing on one side (facing the liquid stream) at the edge of the coupons (round) (Figure 6.2a). The coupons were mounted on a mixing cylinder. When the mixing was stopped and the coupons were sampled, the biofilm started to disrupt, indicating instable biofilm growth. *P. putida* and *R. mucilaginosa* tended to grow at the edges of round, smooth polypropylene coupons but in case of *P. putida* or *R. mucilaginosa* the biofilms were less hetergeneous. For all these reasons *Microbacterium* sp. biofilm was not considered for further testing. Another candidate for Gram-positive bacterium biofilms was *Staphylococcus cohnii* for which no biofilm reactor experiment was conducted.

Figure 6.2. Biofilm of a) *Microbacterium* sp. (14 d post inoculation) strongly grew on only one side of the coupon. b) *P. putida* biofilms (13 d post inoculation). c) Biofilms of *R. mucilaginosa* (5 d pots inoculation) were growing all around the edge of the coupon.

6.3.4 Storage

It could be observed that freezing either with or without any cryoprotectant was the worst storage condition for *P. putida* biofilms with total losses of viability, protein and polysaccharide. The biofilms that were used for the washing test were consequently only stored in glycerol (*P. putida*) or in trehalose (*R. mucilaginosa*) at 4°C because most of the EPS still remained on the coupons and also cells were still viable. However, none of the tested storage conditions was able to preserve biofilms without losses of biomass or impairment of biofilm stucture (Chapter 5). Especially for removal tests it is important that a minimal amount of biofilm remains on the coupons in order to determine differences before and after washing.

Depending on the detection method the detection limits can vary. The staining procedure with crystal violet for the semi-quantitative evaluation of biofilms is less sensitive and needs ca. 10^4 cells on the coupons.

6.3.5 *In situ* washing tests with stored biofilms

For *P. putida* we could observe that with the bleach containing standard detergent more viable cells were removed at the lower and middle position than with the bleach-free color detergent.

Removal tests with *R. mucilaginosa* revealed that depending on the location the reduction of viable cells is much lower (37 - 81%) without chemical forces than in combination of chemical and mechanical stresses (Table 6.3). Under these test conditions, *R. mucilaginosa* also seemed to tolerate chemical stresses better than *P. putida*, leading to the assuption that

the effects of chemical and mechanical stress seem to be strain-dependent. In addition, for *R. mucilaginosa* it was observed that the position "upper back" side led to reduced biofilm removal confirming that less accessible parts (to physical or chemical stress factors) of the washing machine help to promote biofilm formation. Munk et al. (2000) exposed *S. epidermidis* on textiles directly into the washing cycle at 30°C. They observed that bleach-free liquid detergent did not remove the bacteria effectively. Only in presence of bleach containing detergents the number of cells was reduced. Compared to *P. putida* and *R. mucilaginosa*, they were completely exposed to temperature and detergents.

In a second set of experiments the amount of remaining protein and viable cells were compared. Most of the cells of *P. putida* (>98%) were removed after the washing procedure at 20°C and 40°C in prescence of liquid detergent (Table 6.4). For *R. mucilaginosa* washing at 20°C reduced maximal 85% of the cells. More cells could be removed at 40°C (97% in minimum). For *P. putida* the thermal factor was subordinate. Due to changes in the environmental motivation low temperatures are preferred (SKW 2010). The thermal effect is not the primary factor anymore to kill microbes. Janacek et al. (1981) stated that washing temperatures from 20-40°C did not sufficiently remove cells during laundering.

The chemical and physical stress factors mainly affected cell viability. In both cases a large amount of proteins still remained on the coupons after washing (Table 6.4). It can be assumed that besides the proteins also the carbohydrates were still present. This could be an indication for present extracellular polymeric substances (EPS). If a biofilm is not completely removed after washing, the remaining organic compounds of a biofilm could serve as a carbon and energy source (Sutherland 2001) for surviving cells and also as an anchoring site (Neu 1992) for newly introduced microbes.

Table 6.3. Washing machine experiment with 2-weeks stored biofilms of *P. putida* and *R. mucilaginosa* (indicated as % of removal of CFU). The influence of the detergent is given as percentage of removal of CFU (n=3).

Washing temperature	Location in washing machine	No detergent CFU [% of removal]	Persil Color Gel™ CFU [% of removal]	Standard detergent CFU [% of removal]
P. putida				
30°C	Middle	n. d.	87.5	99
	Lower	n. d.	96.5	100
	Upper backside	n. d.	98.5	81.5
R. mucilaginosa				
30°C	Middle	80.5	96.5	n. d.
	Lower	73.5	81.0	n. d.
	Upper backside	36.5	81.0	n. d.
40°C	Middle	n. d.	94	n. d.
	Lower	n. d.	89.5	n. d.
	Upper backside	n. d.	90.5	n. d.

Table 6.4. Removal tests in household washing machines with 2-weeks stored *P. putida* and *R. mucilaginosa with* Persil color Gel^{TM} at 20°C and 40°C, respectively. The removal is indicated as percentage of removal of CFU and total protein.

Washing temperature	Location in washing machine	CFU [% of removal]	Total proteins [% of removal]
P. putida			
20°C	Middle	98.5	17.0
	Lower	98.5	47.0
	Upper backside	98.5	47.0
40°C	Middle	98.5	33.5
	Lower	99.9	23.0
	Upper backside	99.8	0
R. mucilaginosa			
20°C	Middle	85.5	26.5
	Lower	68.0	10.0
	Upper backside	82.0	28.5
40°C	Middle	99.0	34.0
	Lower	99.5	42.0
	Upper backside	97.0	14.0

6.3.6 Simulated transport conditions

In this experiment the stability of stored biofilms under transport conditions was evaluated. In the presence of 0.9% NaCl solution or phosphate buffer the stainless steel carriers started to rust. Trehalose or no transport protectant was more appropriate for the carriers. The initial number of biofilm amount after 2-weeks storage was rather low with 10^4 cells per coupon. After the transport simulation, reduction of 2-log could be observed for both strains under high shaking conditions. As expected, high shaking of the biofilms led to the detachment of *P. putida* model biofilms. However, *P. putida* also got removed from the coupon even without shaking.

In case of *R. mucilaginosa* the different shaking protocols led to more than 1-log reduction, when it was stored in presence of trehalose. Storage without cryoprotectant reduced the amount of cells by 1-log indicating that it is more appropriate not to use protectants although trehalose was described as an important endogenenous protectant in the survival of yeasts and membrane stabilizer (Bolat 2008).

All blanks (control coupons) with one exception were colonized with 10^1 - 10^3 cells cm^{-2}. This clearly indicated a cross-contamination during shaking. The blanks and in some cases coupons with *P. putida* were colonized by *R. mucilaginosa*. Contaminations with *P. putida* were mainly observed at low shaking conditions or without shaking (Figure 6.3), conditions for which *R. mucilaginosa* was poorly detecting. This indicates that the stored cells are viable after storage and are able to re-colonize surfaces independent of the prescence of a cryoprotectant.

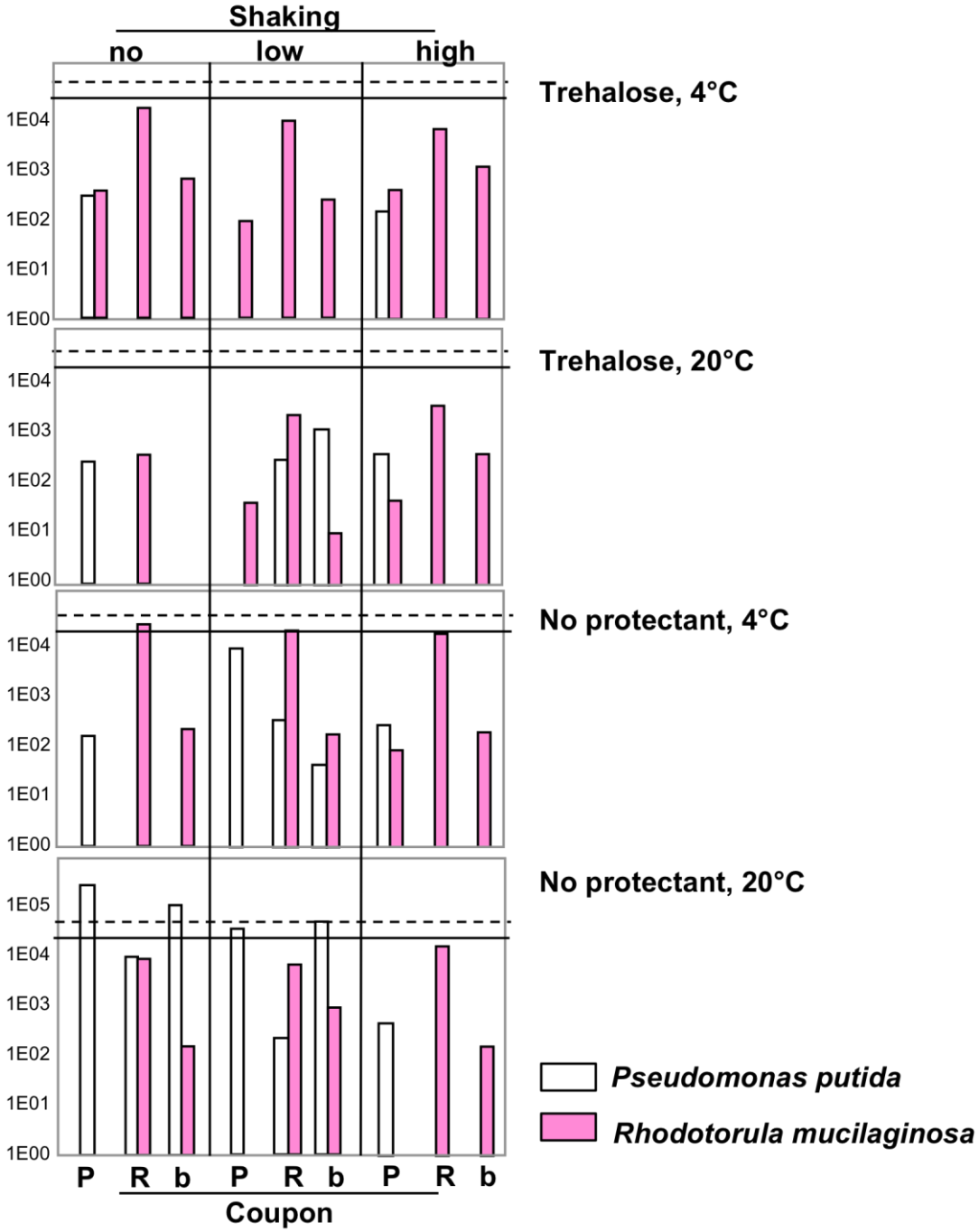


Figure 6.3. Effect of simulated transport conditions on *P. putida* and *R. mucilaginosa* biofilm cell viability stored in trehalose or without protective agent at 4°C and 20°C for two weeks. P: *Pseudomonas putida*, R: *Rhodotorula mucilaginosa* b: blank (control), plain line: initial count for *P. putida*; dashed line: initial count for *R. mucilaginosa*.

6.3.7 Principle of the application of the model biofilm and staining procedure

The model biofilm and the staining procedure are parts of a kit to determine the removal efficiency of household washing machines. The principle of the application is as follows: The enduser mounts the model biofilm at representative locations (i.e. prone to biofilm formation) around the outer drum of the washing machine. A washing cycle is applied and the remaining biofilm after the treatment is quantified either by semi-quantitative crystal violet staining and/or by quantification of total protein and polysaccharide amounts, optical density, and CFU (Figure 6.4).

The application of this test system is not limited to household washing machines. This standardized biofilm can be adapted to the food, medical and chemical industries. In particular, the biofilm can be used to study the efficacy of detergents, surfactants or disinfectants. The model can also be beneficial to adjust the efficiency-dosage especially in terms of biofilm removal and help to develop new cleaning procedures.

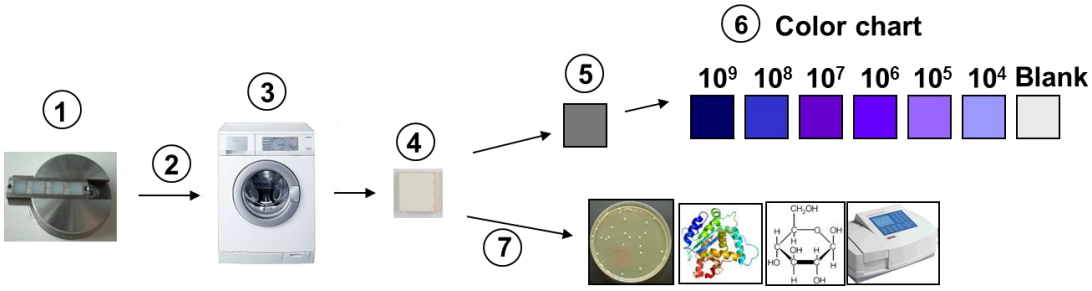


Figure 6.4. Work flow of the biofilm produced in the bioreactor and used to test biofilm removal in household washing machines. 1) assembly of different single species biofilms together with a blank as negative control on a holder, 2) mounting of the carrier in the washing machine, 3) washing cycle, 4) removal of coupons from the washing machine, 5) qualitative quantification via staining remaining biofilm with crystal violet, 6) comparison of color with a color chart, 7) quantitative determination of biofilm via viable cell count, protein and polysaccharide quantification and optical density.

6.4 Conclusions

The result of the cultivations in the designed reactor system was a model biofilm that can be used as a test system to determine the removal efficiency of biofilms in household washing machines. Together with the staining procedure and the methods for biofilm quantification, this builds a powerful tool to determine easily biofilm formation and its

removal. The model biofilm was designed for household washing machines and could also be applied in other systems (cooling system, dishwasher) or also to test the efficiency of antimicrobial agents on thicker and older biofilms.

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7. Conclusions and Outlook

7.1 Biofilms – Can the problem be washed away?

7.1.1 The future of the standardized biofilm model

Biofilms in household washing machines can damage the material, decrease the hygienic performance, lead to contamination of the laundry or to malodor production. Household washing machines are prone to biofilm formation like any other fluid or humid system and are commonly colonized by soil or waterborne bacteria, yeasts or fungal species which enter the system either with the water used for washing or with the dirty laundry (e.g. skin or fecal microorganisms). Energy-saving measures and increased usage of bleach-free detergents facilitate the establishment of biofilms. Washing machine manufactures are aware of this problem and started developing measures against biofilm formation using ultrasound, specific hygienic programs or release of silver ions. However, the efficiency of these methods has not been determined due to lack of an appropriate removal test based on model biofilms.

Biofilms in household washing machines. The fact that microorganisms are able to grow in household washing machines is already well-known. Malodor was the most predominant appereance indicating microbial activity. Besides odor, biocorrosion contributed to damage of the machines. But until now, the microorganisms building the microbial community in household washing machine were rather unexplored. This Ph.D. thesis was the first attept, to investigate the microbial composition and to evaluate the microorganisms that are predominantly found in washing machines (**Chapter 2**). For this purpose, household washing machines were dismantled and analyzed for existence of microorganisms. The findings revealed that household washing machines harbored a wide variety of microbes (over 90 bacterial and fungal isolates were identified) of which also 30% belonged to opportunistic pathogens. Moreover, depending the origin of the washing machines, the composition of the microbiota slightly differed.

Although a plethora of microorganisms has been isolated, it was not representative of the actual microbial diversity in household washing machines. The results are based on recultivation of mesophilic, fast-growing, aerobic, microorganisms, thus exposing the microorganisms to a selection pressure. To really get an image of the microbial diversity 16S rRNA and 18S rRNA analysis would be more adequate method. Because we found

more fungal isolates from washing machines from South Korea than from other countries we asked ourselves, if geographical postions, climate, water quality and laundry behavior have an impact on the microbial community.

The impressive amount of opportunistic pathogens found in household washing machines automatically provoked the question: Could household washing machines be a potential source of infections? We assumed that the household washing machine as source of infection of the endusers is small because the biofilms tended to grow on parts which were less accessible for direct cleaning by the enduser and well-protected from chemical and mechanical forces during the washing cycle and detached cells that could enter the drum are usually removed or killed during the washing cycle. Although no reports are available a health risk cannot be entirely excluded. Health risk is more likely for workers that have to dismantle washing machines and are directly exposed to the biofilms.

Even though, all these open questions are interesting and are worth to draw ones attention, they were not addressed for further investigations because we were primarly focusing on finding ideal candidates for tailored model biofilm that can be used for efficiency tests of washing machines and laundry detergents.

To support the hypothesis that environmentally isolated strains are better candidates for biofilm models than reference strains, we exposed *P. putida* isolated from a washing machine and its reference strain to different concentrations of a standard detergent (ICE-A*). The results indicated that the isolate was more resistant than the reference strain in terms of biomass. At the recommended concentration of ICE-A* for washing (7 g L^{-1}), both strains were eradicated and no viable cells could be determined. However, the exopolymeric substances (EPS) and some cell debris remained on the surface and therefore, could further serve as source of nutrients or anchoring point for new biofilm forming cells. These results suggested, that the available commercial detergents are far away from being a definitive solution of removing biofilms from surfaces and that only a combined treatment (with mechanical force, water and temperature) against both viable cells and EPS would be the most efficient solution.

Biofilm model. *Rhodotorula mucilaginosa* and *Pseudomonas putida* were chosen for the repeatable cultivation of single-species biofilms in a modified bench-top reactor due to their biofilm forming ability, cultivability and unproblematic handling.

As described in **Chapter 3,** a biofilm bioreactor was set up where coupons with different materials were mounted on a rotating cylinder in the center of the culture broth. The main purpose of the cylinder was to ensure a homogenous distribution of shear and nutrients to enable an even biofilm formation on all test coupons. A repeatable biofilm could be achieved with *Rhodotorula mucilaginosa* on smooth polypropylene (PP) surfaces originating from a household washing machine. Although more biofilm could be formed on rougher PP surfaces, the variability between the samples increased and also the tendency for sloughing was enhanced. Interestingly, dye-casted PP coupons with a pre-defined roughness did not show similar fouling properties. Also the decrease of hydrophobicity with plasma treatment, which is described to enhance cell attachment of mammalian cells did not promote microbial attachment. In our high shear system, the surface structure played a central role whereas the hydorphilicity/hydrophobicity of the substratum for cell attachment was not of significant importance.

In contrast to *R. mucilaginosa*, *P. putida* formed an irregular biofilm that was not repeatable in its structure and amount. Biofilm gradients were observed from top to bottom of the reactor due to the location of the nutrient supply port. This finding suggests that homogenous distribution of nutrients within the reactor is an important factor affecting biofilm formation **(Chapter 4)**.

Although the presented system could be used for cultivation of standardized biofilms of *R. mucilaginosa*, the cultivation conditions for *P. putida* still leaves room for improvement in terms of material modification and reactor set-up.

Biofilm storage. Agar plates kept at ambient temperature were the sole place to store microorganisms for a long time before refrigerators and freezers were available and are until today routinely used for short time storage. The possibility to keep microorganisms at very low temperatures (down to -85°C) increased their shelf-life up to 10 years (Bast 2001).

Indeed, biofilms are more difficult to store because they are composed of cells and extracellular matrix. However, prolongation of shelf life makes biofilm increases the flexibility of the experimenter to perform e.g. anti-biofilm test whenever one wants.

In our study, biofilms of *P. putida* and *R mucilaginosa* were stored in presence of different cryo protectants (glycerol, trehalose, hydroxyectoin or no additive) at different temperatures (Chapter 5). Although storage was possible at almost all conditions, we found that typical temperatures for long-term storage (-20°C and -80°C) were not appropriate

because they disrupted the biofilm and resulted in a loss of viability. Also freeze-drying was not an alternative. The results indicated that humidity and elevated temperatures are essential to keep biofilms intact.
The results suggested that the most appropriate conditions for both organisms for long-term storage (2 weeks – 2 months) were at room temperature or 4°C in presence of trehalose. This test conditions were considered as optimal because sufficient cells and organic material remained on the test coupons for testing biofilm removal efficiency in household washing machines.

Primary application of standardized model biofilm. The main goal of the thesis was to provide a model biofilm for the assessment of the removal efficiency of household washing machines (**Chapter 6).** The most important parameters were identifying the appropriate model strains and materials to produce biofilms in a repeatable manner. These should be representative for the situation in a household washing machine. We also aimed to quantifying the removal efficiency of household washing machines. Staining experiments were performed to facilitate qualitative quantification of the biofilm and its removal. For detection purposes, crystal violet in combination with white PP coupons gave the largest contrasts for both fouled and unfouled material. The biofilm model together with the quantification method, enables the household washing machine manufacturer to estimate the washing performance of a specific program as well as the washing efficiency of washing detergents in a relatively fast way. Often for industrial applications, fast and simple quantification methods are mostly desired. More sophisticated quantification methods depend on a well-equipped (scientific) laboratory and lab technicians.

7.1.2 The appropriate reactor system for biofilm formation

Using the appropriate tools and cultivation system for biofilm production is very essential. The development of biofilms is affected by many different factors (e.g. species, surface material, nutrients, shear forces, pH). Therefore, the control of as many parameters should be given. The presented reactor system has the great advantage to control several of the factors mentioned above.
The modified bioreactor was chosen based on versatile advantages compared to other commercially available biofilm reactor like flow cells or annular disk reactors. Unidirectional flow cells like Robbins devices create heterogeneous biofilms due to

gradients of nutrients. (i.e. high nutrient availability at the entrance and low nutrient availability at the exit of the reactor). Annular reactor are similar to the customized biofilm reactor, however, providing less biofilm samples per production process and being less handy due to limited autoclaving possibility.

The customized cylinder mixes the nutrients homogenously (to prevent spatial gradients) within the reactor and exposes the biofilms to the same shear forces.

In the presented study, spatial gradient from top to bottom of biofilm prodcution are still an issue, although they could successfully be reduced by placing the medium inlet port in the middle instead of introducing the medium from the surface of the culture broth (Chapter 3). The theoretical output of the reactor system provides up to 120 samples per reactor. Other commercially available reactors (e.g. annular disk reactor or the rotating disk reactor) provide less biofilm samples (ca. 24 coupons). Unfortunately, due to gradient formation, the outermost coupons were not taken into account, resulting in a loss of 12-24 coupons per bioprocess. Considering commmercial biofilm production rejects of 12.5% is very high. At the moment, the biofilm reactor represents a prototype to analyze sources of problems during cultivation and therefore, the yield is highly acceptable. For high-throughput biofilm production, up-scalling of the bioprocess will be necessary. The feasability of repeatable biofilm production, with e.g. 20-L fermenters, needs to re-evaluated due to the varying dimension and cannot be directly extrapolated.

The possibility to continuously cultivate biofilm in the customized biofilm reactor, gives also a chance to harvest biofilms before reaching steady-state of biofilm assembly and dispersal. In this phase, biofilms are still relatively young, producing only little EPS (microcolonies). The response towards antimicrobials could deviate from those of mature biofilms.

7.1.3 The future of the standardized biofilm model

Overall, we demonstrated that the concept of tailored biofilm production in biofilm reactors could be accomplished. To achieve model biofilms, the complexity of natural biofilms was reduced, considering only single-species biofilm models on one type of material (PP) to test repeatability of the bioprocess and biofilm formation. The obtained results for repeatable biofilm formation, in particular, of *R. mucilaginosa* biofilms are promising. However, the improvement of the cultivation and biofilm quantification should be considered further.

Hence, one of the biggest problems that still needs to be solved for the **optimization of the bioprocess** is the further decrease of variability of samples between reactors experiments. Because we applied relatively simple and cheap **quantification protocols** (so that other laboratories can easily reproduce it) which demand "manual handling", it is also very likely that sampling errors occured, increasing the variability between the samples. Automatization of polysaccharide and protein quantification and flow cytometry for cell counts might rather be an accurate but expensive countermeasure for industrial biofilm production.
Polypropylene was the most appropriate **material** to start optimizing the cultivation process and the reactor set-up because it was easily colonized by cells and gave the largest contrast between fouled and unfouled surfaces with crystal violett. Biofilm production is not limited to polypropylene. As biofilms are growing on possibly all available materials, it is also of great interest to use other types of plastics, metals, rubber or medically relevant material for implants or catheters.

A challenging perspective is the development of a **mixed biofilm** which is the regular case in natural environments. In our studies, biofilm formation was limited to single species biofilms in order to decrease the number of manageable parameters. The selection of the right strains that will be cultivated togehter has to be well considered because depending on the combination of different species the relationship between the microorganism can be either synergistic or antagonistic. A possible synergistic effect is the increased biomass as well as overall resistance of the biofilm when different species were cultivated together in comparison to single-species biofilms. The effect is still unknown, speculating that species-specific physical interactions, extracellular secreted factors and less specific interaction may play an important role favoring biofilm growth. On the other hand, antagonistic effects are also likely due to competition in terms of nutrients resulting in a pre-dominant strain as well as the production of virulence factors (bacteriotoxins) that kill or inhibit growth of the other strains (Burmolle et al. 2006). Establishment of a mixed species biofilm is more demanding because this also raises the question, if the spatial distribution, the cell and biomass fraction of two or more strains can be achieved in a repeatable manner. For cultivation purposes, it might also be important how to inoculate the system, if sequentially specific strains are depending on a primary colonizer before being able to grow in a the biofilm (secondary colonizer) or all strains at once enabling the strains to organize themselves.

7.2 Tailor-made model biofilms - Applications and potentials

In this thesis, only one application of a model biofilm - the determination of removal efficiency of household washing machines including washing detergent - has been presented. The need for a standardized model biofilms is indisputable. However, a general model that fits all the requirements of all systems colonized by biofilms is not possible.
Standardized model biofilms find their potentials in diverse fields (e.g. food and pharmaceutical industry, ecology and public health). They have their application in the evaluation and optimization of cleaning procedures, industrial plant design, quality control of goods, ecological analysis (e.g. soil, water), screening and testing of antimicrobial agents and the adjustment of standard procedures (e.g. ASTM, ISO).

7.2.1. Model biofilms for evaluation of cleaning and decotamination procedures

In many different industries (e.g. food and pharmeceutical industry) as well as in medical and healthcare system, hygiene is highly crucial to diminish the risk contamination and consequently quality impairement or infections.
Permanent eradication of biofilms on a fouled surface will most probably never be achieved but regular cleaning and disinfection can control and minimize biofilm formation. Therefore, it is important to evaluate the efficiency of different cleaning procedures. The optimal cleaning procedures remove all organic materials including the EPS as well as cells to minimize recolonization followed in combination with a decontamination step to kill the cells.

Cleaning and decontamination in medicine and healthcare. Effective cleaning (removal of soil and microorganism of biofilms) is not only important in industrial process but also in healthcare system. Reusable medical devices such as invasive endoscopes or scalpels, dental tools and water lines as well as surfaces of healthcare facilities have to be regularly cleaned and disinfected to prevent infections or cross-contamination between patients.
Cleaning of reusable devices undergoes multiple cleaning steps including manual and/or mechanical treatment (among others ultrasound treatment) before sterilization. For the cleaning solution a mixture of detergent, disinfectant and enzymes is usually applied to clean the device. However, the mixture has to be adjusted as the effect of different

chemicals can be neutralized. In other protocols, different chemicals had to be used consecutively in order to be effective.

Model biofilms could be used for preliminary studies of testing the efficiency of cleaning protocols before testing it on the medical devices as they are relatively expensive for having several samples. Cleaning of medical devices can be relatively difficult because the design of the devices are rather complex and should therefore be tested with the device itself.

Cleaning and decontamination in industrial settings. Stable and mature biofilms are problematic in industries as they often lead to spoilage of the products (van Houdt and Michiels 2010). In different **industries** (e.g. food and beverage processing, pharmaceutical and biotechnological industry), closed processing systems consisting of pipes, heat exchangers or tanks are commonly used. The pipes and tanks are often hardly accessible for manual cleaning and if manual cleaning is possible, it may lead to prolonged downtime. Therefore, automatic clean-in-place (CIP) systems are preferred. They have the advantage of reducing cleaning cycles using a combination of mechanical (shear force of flowing water to rinse the system), thermal and chemical force (sanitation with a disinfectant). However, it has been shown that biofilm still remained after cleaning (Wong 1998). Regular cleaning and disinfection prevent microbial contamination of the product or reduce damage of the units. Often, not only insufficient cleaning procedures enable formation and persistence of biofilms, but also the physical design of closed systems can influence biofilm formation. Areas of reduced fluid exchange with the cleaning solution as well as dead ends favor biofilm formation because of diminished accessibility. Computational fluid dynamics (CFD) simulations are performed during the design phase (http://www.pathogencombat.com). The computer software, in combination with the model biofilms placed in such structures could also evaluate the design of the process plant during the developmental phase of new industrial plants.

7.2.2 Model biofilms as biosensors in industry and ecology

After industrial processing, the end product (e.g. food, drinking water) is regularly controlled to meet the given standards of product quality, product safety and public health. Although cleaning or disinfection procedures in industry, healthcare or water treatment are effective and reduce contamination when the correct measures are followed (e.g.

guidelines for best hygienic performance), contaminations cannot be completely excluded. Therefore, model biofilms could be used as biosensors (a sensor based on a biological component such as microorganisms) to indicate the presence of specific contaminants (toxic elements, microbial species or virulence factors) as they react with the contaminant. Besides product quality, the application of the model biofilm can also be extended to detect environmental pollutants in waste products, soil or waters (e.g. heavy metals and petrolium). The principle of bacterial biosensors has already been developed to detect arsenic and arsenate in drinking water. Arsenic intoxication is a large problem in several countries (e.g. Bangladesh). The biosensor consisting of genetically modified bacteria are fixed on a paper strip and start to produce a blue color if arsenic is present in the water sample. The color intensity is proportional to the arsenic concentration. The advantage of the arsenic biosensor is that it enables cheap and simple field application in comparison to classical chemical field detection methods (van der Meer and Stocker 2003).

7.2.3 Screening and evaluation of (new) antimicrobial agents

The chemical and pharmaceutical industry are permanently looking for new potential antimicrobial agents to reduce or prevent biofilm formation. Standardized model biofilms could serve as screening tools for new compounds (e.g. synthetic or natural products) on their anti-biofilm potential (e.g. quorum-sensing inhibitors and enzymes to disrupt established biofilms or compounds with a microcidal activity) as well as to determine dosage and exposure time for optimal antimicrobial effect.

So far, most available standards dealing with testing antimicrobial activity of a compound is performed with i) agar diffusion tests, where the zone of inhibition is decisive for the analysis of the susceptibility of a determined reference strain or ii) with liquid cultures for determination of minimal inhibitory concentration. The results of both test can not be extrapolated to biofilms because planktonic cells and colonies on agar plates are not physiologically identical to cells in biofilms. Planktonic cells are easier to handle and may give clearer results, e.g. for testing antibiotic and biocides. However, this may falsify the outcome considerably as it is known that cells in a biofilm are up to 1000 times more tolerant towards antimicrobial agents (Mah and O'Toole 2001). Therefore, it is advantageous to use standardized biofilms and also to adjust existing norms for the evaluation of antimicrobial agents.

7.2.4 Commercialization of model biofilms

Currently, only three standard methods are available (ASTM Method E2647, E2562 and E2196) to produce repeatable biofilms of *Pseudomonas aeruginosa* produce in a drip flow biofilm reactor, CDC biofilm reactor and rotating disk reactor. Goeres and co-workers developed the standard method for repeatable biofilms in drip flow reactors with the aim to use these biofilms for testing disinfectants under real application conditions (http://www.astm.org).

These standards have the disadvantage that they are optimized for a single microorganism and that each laboratory or company that wants to test disinfectants or optimize a cleaning procedure is obliged to produce their own biofilms before they can perform the actual experiments and optimization processes. This requires that the laboratories and companies are equipped with the appropriate facilities and trained personnel.

Therefore, commercialization of biofilms would be a practical solution, providing laboratories and companies with tailored model biofilm according to their specific application, which are ready to use for antimicrobial and cleaining studies. This idea has already been applied in tissue engineering, where specialized companies produce three-dimensional human skin models that are applied for testing cosmetics or pharmaceutical agents on their compatibility or effect on skin (http://www.skinethic.com).

However, the commercialization of standardized model biofilm is still a distant prospect because so far, the main production processes are still performed at a small-scale and problems of biofilm storage and transport without loss of biomass or cell viability still need to be addressed.

7.2.5 Personal comments

In the future, the need for standardized model biofilms will increase due to the global biofilm problem in medicine, industry and environment. It is unrealistic to assume that undesired biofilms will ever be eradicated or avoided. Therefore, the efficient control of biofilm growth needs to be addressed instead. At the moment, the field of applicable biofilm especially for *in situ* application under real use conditions is relatively unexplored.

The idea of producing a standardized biofilm that can be achieved in a repeatable and reproducible manner is very challenging, notably being aware of dealing with complex communities of microorganisms that are controlled by numerous parameters.

In my opinion, this doctoral thesis is a essential contribution to satisfy the need for a standardized model biofilm, still leaving space for further improvement. I am confident that the problems that occured during cultivation and storage can be diminished in the future and that commercially available biofilms will be the standard in applied biofilm research and in industries encountering problematic biofilms.

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Acknowledgments

During the last three years of my Ph.D studies several people helped me out with my thesis. At this point I would like to thank all those people.

I would especially like to thank Dr. Laurie Mauclaire for her great supervision of my thesis and having confidence in me as well as Dr. Manfred Zinn for his support.
Dr. Linda Thöny-Meyer I would like to thank her for giving me the opportunity of doing my PhD thesis in her group.
Many thanks go to Prof. Dr. Leo Eberl and Prof. Dr. Jakob Pernthaler for being part of my PhD committee and Prof. Dr. Jan-Roeloff van der Meer for reading my thesis.

Many thanks also go to Daniel Fäh, Caroline Amberg, Bettina Lanz and Monika Brägger from ETM who enabled this project together with CTI for the financial support.
I would also like to thank Kathrin Grieder, Aline Hunziker and Fiona Wüthrich for their support in the laboratory and Dr. Bruno Wampfler for all the statistical discussions.
I would also like to thank Stéphanie Follonier, Bernhard Henes, Sandro Dilettoso, Daniela Bischof Boesch, Manuel Bühler, Christian Müller, Sylvaine Le Meur and Valérie Roux for being such nice and interesting office companions. I had lots of fun with you and laughed a lot in and outside the office.
I also appreciated very much that I get to know Renate Reiss. She knew how to cheer me up when times were less nice, and also for her time, company and friendship in the last two years. Through her I know now that St. Gallen rocks!
And also many thanks to all boys and girls of the Biomaterials group and former members for their company, interesting discussions during the coffee breaks and all the delicious cakes and Gipfeli.
Further I want to say “thank you” to Patricia Gavilano and Anna Lena Dätwyler that they did not forget about me although I was not able to see them very often in the last three years.
I am very grateful that Juan Miguel Escobar was always there when I needed him, his patience and for having an open ear.
Last but not least I would like to thank my family, especially my parents Mercedes and Alfred who always believed in me.

Curriculum vitae

Personal Information

Name	GATTLEN
First names	Jasmin Claudia
Date of birth	07 May 1982
Home town / canton	Bürchen VS

Education

1997 – 2002	Kantonsschule Stadelhofen, Zurich, Typus B
2002 – 2007	Biology, University of Zurich
	Biology, Microbiology
	Master of Science in Biology, Microbiology Prof. Dr. Leo Eberl, Department of Microbiology, Institute of Plant Biology, University of Zurich, Switzerland. Thesis title: "Global analysis of gene expression in *Burkholderia cenocepacia* H111 in response to iron limitation by proteomics"
2007	Internship at the States Secretariat for Economic Affairs (Seco)
2008 - 2010	**PhD Student,** employed by EMPA, St. Gallen, immatriculated at the University of Zurich
2011 – present	**Research assistant**, employed by ZHAW, Wädenswil

Publication

Gattlen J, Amberg C, Zinn M, Mauclaire L. 2010. Biofilms isolated from washing machines coming from three continents and their tolerance to a standard detergent. Biofouling 26(8):873-882.

Presentations/Talks

2010. Empa PhD Symposium, Dübendorf, Switzerland. Poster: "Biofilm in household washing machines or how to evaluate its removal".

2010. 5th International Summer School on Advanced Biotechnology. Santa Margherita di Belice, Italy. Talk: "Production of a model biofilm for testing biofilm removal".

2010. 69th SSM Meeting of the Swiss Society for Microbiology, Zurich, Switzerland. Poster: "Kit for evaluating biofilm removal in household washing machines".

2009. Empa PhD Symposium, Dübendorf, Switzerland. Talk: "Model biofilm for testing washing efficiency of household washing machines".

2009. Eurobiofilm congress, Rome, Italy. Poster: “Repeatable biofilm formation in custom-made biofilm reactor under controlled cultivation conditions”.

Grants

International Society for Microbial Ecology (ISME-13) follow up in Seattle
The ISME Travel Awards for Young Scientists, August 22-27 2010 Seattle, WA, USA.